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T CELL RECEPTOR REPERTOIRE TO POLY-18 AND Mls-1^{a}: PARALLELS BETWEEN A PEPTIDE ANTIGEN AND SUPERANTIGEN RECOGNITION

BY

PATRICK DAVID KILGANNON

A thesis

submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of

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IN

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Patrick D. Kilgannon
11428-53 avenue
Edmonton, Alberta
T6H 0S8

Date: Nov 26, 1990

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled

T CELL RECEPTOR REPERTOIRE TO POLY-18 AND Mls-1^a: PARALLELS BETWEEN A PEPTIDE ANTIGEN AND SUPERANTIGEN RECOGNITION

Submitted by

PATRICK DAVID KILGANNON

In partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

IN MEDICAL SCIENCES (IMMUNOLOGY)

Dr. B. Singh (Co-supervisor)

Dr. A. Fotedar (Co-supervisor)

Dr. T. Wegmann

Dr. V. Paetkau

Dr. D. McKean

Date: Nov 26, 94

DEDICATION

To Karen

For your endless support and encouragement,
You've kept my feet on the ground
Thank you

To Trevor

I've been in school your whole short life,
You are my strength
Thank you

Abstract

T cells recognize processed antigen in association with molecules of the major histocompatibility complex (MHC) on the surface of antigen presenting cells (APC's). This interaction is mediated through the T cell receptor (TCR) α and β chains. We have attempted to determine the relative contribution of the different gene segments that make up each chain of the TCR for the recognition of 2 antigens, poly-18 and Mls-1^a.

We have characterized TCR gene usage in the BALB/c T cell response to the synthetic peptide antigen poly-18 [poly EYK$(EYA)_5$]. Our analysis of the T cell response to this antigen shows 2 V genes dominating the T cell repertoire, Vα11 and Vβ6. In a panel of 16 poly-18 specific and I-A^d restricted T cell hybridomas 50% use Vβ6 while another nonoverlapping group (25%) uses Vα11. A large number of other chains can pair with the dominant V gene chains in the poly-18 response. The junctional diversity and J region usage associated with the dominant V genes was assessed using the polymerase chain reaction. These sequences were amplified and cloned from appropriate T cell hybridomas and a number of bulk T cell lines specific for poly-18. The junctional diversity and J region usage associated with the dominant V genes are extremely heterogeneous. These observations parallel some features of the superantigen systems, suggesting that recognition of peptide antigens can potentially be conferred primarily by individual V regions.

In addition, we have addressed a number of issues related to recognition of Mls-1^a by poly-18 specific T cells that express Vβ6.

The correlation of Vβ6 expression with Mls-1^a reactivity is well documented but the exceptions to this correlation have not been characterized. From a panel of poly-18 specific Vβ6+ T cell hybridomas, we have identified a number of Mls-1^a nonreactive T cells. The TCR Vα gene usage and β chain junctional diversity in these hybridomas was assessed. The existence of these cells in the poly-18 T cell repertoire suggested that this might be an appropriate antigen to amplify rare Vβ6 T cells that may have escaped deletion in a Mls-1^a mouse strain. We have succeeded in generating poly-18 specific T cell lines from DBA/2 that contain Vβ6+ T cells. The junctional diversity and Jβ region usage in these T cells has been assessed using PCR to amplify and clone these sequences from the bulk T cell line RNA. There is a wide degree of junctional diversity and Jβ usage associated with the failure to be deleted in these mice. Our results suggest that some Vβ6 T cells do escape thymic deletion and are immunocompetent. The inability of these Vβ6 T cells to recognize Mls-1^a may be caused by a number of elements in the TCR.

The parallels between T cell receptor gene usage in the recognition of poly-18 and Mls-1^a suggest that peptide and superantigen recognition may not be fundamentally different types of interactions. Rather, when viewed in the context of numerous antigen systems, a broad range of response patterns emerge.

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Saving the very best for the last, I must thank my parents. To my father, I thank you for teaching me the value of hard work, be it physical or mental. To my dear mother, you have always been my source of inspiration, thank you.

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List of Abbreviations

Ab	Antibody
AED	*N* -iodoacetyl-sulfonic-naphthyl-ethylene-diamine
APC	Antigen presenting cell
bp	base pair
C	Constant region
CDR	Complementarity-determining regions
CTL	Cytotoxic T lymphocyte
D	Diversity region
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
EAE	Experimental autoimmune encephalomyelitis
FACS	Fluorescence activated cell sorter
H chain	Heavy chain
HLA	Human leukocyte antigen
Ig	Immunoglobulin
Ir	Immune response
J	Joining region
Kb	Kilobase
Kd	Kilodalton
L chain	Light chain
MBP	Myelin basic protein
MHC	Major histocompatibility complex

MLR	Mixed lymphocyte reaction
Mls	Minor stimulatory
MW	Molecular weight
OVA	Ovalbumin
pABA	p-azobenzenearsonate
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RTase	Reverse transcriptase
SDS	Sodium dodecasulphate
SE	Staphylococcal enterotoxins
TCR	T cell receptor
TNP	2, 4, 6-trinitrophenyl
V	Variable region

Chapter 1

Introduction

The hallmark of the mammalian immune system is the ability to discriminate foreign antigen from self molecules. The specificity associated with the immune system is central to the efficient execution of an immune response. The immune response to a given antigen is induced by the antigen, yet feedback mechanisms exist to control the magnitude and duration of a response. Should the immune system come in contact with an antigen after the primary response, the secondary response is faster and stronger. Thus memory is another distinguishing feature of the immune system. All together these 4 features of the immune system (specificity, inducibility, feedback control, and immunologic memory) define many of the parameters of an immune response.

There are two interconnected arms of the immune system that are responsible for initiating antigen specific immune responses. Antigen specific receptors on the surface of two related lymphoid cell types allow contact with the foreign molecules. The humoral immune response is mediated through antigen specific receptors called antibodies (Ab) or immunoglobulin (Ig) molecules that are on the surface of, or secreted by, B cells. The cellular response is carried out by T cells and the specificity is conferred by the T cell receptor (TCR). There are two types of T cell receptors, α/β and γ/δ. Unless otherwise stated, this review will focus on the α/β T cell receptor. The antigen specific receptors on both B and T cells are clonally expressed. Therefore each cell expresses receptors with only one

specificity. The clonal selection theory, put forward by Burnet and Jerne in the late 50's, is based on the premise that each cell possesses only one receptor specificity (1). Cells with the appropriate specificity contact antigen via their receptor and are induced to proliferate and initiate effector functions. This clonal expansion in response to an antigen manifests itself as a rapid, and specific attack on the invading pathogen. The ability of the immune system to mount an even more aggressive attack upon secondary stimulation is a result of a large number of antigen specific cells still in circulation from the primary response. While the idea was conceived for B cells and the antibody responses associated with them, the principle of clonal selection applies directly to T cells and the T cell receptor as well. Since that time a great deal of information has been gathered on antibodies, T cell receptors and the genes that encode them.

Antibodies and T cell receptors share many structural and genetic features. Both are members of the immunoglobulin supergene family (2). This is a group of related multimember and single copy genes. The prototypical members, the immunoglobulins, are constructed of a number of homology units. Each unit is approximately 110 amino acids long with an internal disulfide bond and a characteristic folding pattern (3). Antibodies and T cell receptors are both heterodimeric molecules. Each chain is composed of variable and constant regions. The variable region is made up of a number of smaller gene segments. The mature genes are derived from a process called somatic gene rearrangement. This process will be discussed in greater detail in a subsequent section.

T cell receptors and antibodies do differ from each other in 3 very significant ways. Firstly, antibodies recognize whole soluble antigen while T cell receptor recognition requires a processed or partially degraded antigen (4). Secondly, T cell receptor recognizes the processed antigen as presented by a histocompatible antigen presenting cell (APC) (5). Thus unlike B cells that bind soluble antigen, T cells recognize antigen in an MHC restricted manner. Finally, T cells mature in the thymus where selection (positive or negative) of thymocytes based on their TCR expression takes place (6). No equivelant selection process is known to occur in B cell development.

Antigen processing involves binding of the antigen to the surface of the APC, internalization into lysosomal vesicles, partial degradation by acid hydrolysis and reexpression of the antigenic fragments on the surface (4). A number of observations led to our current understanding of antigen processing. T cells are unable to respond to whole protein antigens but are able to recognize proteolytic fragments or synthetic peptides of the antigen. A lag time of 45-60 minutes is required from the time the antigen is in contact with the APC to the time it is available to stimulate T cells. Some antigen was presumed to be internalized as it was unable to be degraded with proteolytic enzyme treatment. Lysosomotropic agents like ammonia and chloroquine inhibit the acid hydrolysis of proteins by raising the lysosomal pH from 4.5 to 6-7. These agents inhibited the ability of the APC to effectively process and present antigen to T cells.

T cell restriction is a result of the T cell receptor interacting with determinants on both the processed antigen and the self MHC molecule (5). A trimolecular complex of TCR, MHC and antigen is therefore thought to exist. Antigen-MHC interactions are thought to precede contact by the TCR. The importance of this interaction will be discussed in a section on determinant selection. Detailed information about the contact residues on each molecule is beginning to emerge. Molecular models of the complex based on structural information will be discussed in a subsequent section. Which MHC molecule an antigen is presented on appears to depend on the processing pathway. Intracellular antigens such as viral proteins are presented on class I MHC molecules while antigens taken in from outside the APC are presented on class II MHC molecules. Accessory molecules on T cells, CD8 and CD4, may stabilize the interaction with class I and II respectively, thereby defining T cell restriction subsets.

Thymic selection generates peripheral T cells that recognize antigen in association with self MHC molecules, yet are not autoreactive. At least two selection steps are postulated to occur in the thymus (6). Negative selection of strongly autoreactive T cells appears to involve bone marrow derived APC's. Positive selection of thymocytes capable of weak interactions with self MHC antigens is thought to be mediated by cells of the thymic epithelium. Those thymocytes not positively selected likely undergo programmed cell death. These processes have a dramatic effect on the size and composition of the peripheral T cell repertoire.

The focus of this thesis is T cell receptor recognition of antigen/MHC. The preceding preamble is intended to cover some of

the current topics in immunology that have direct bearing on the T cell receptor field. The remainder of this review will concentrate on the αβ T cell receptor; the molecules and genes that encode it and the structure/function studies that have attempted to gain insight into the manner in which the it binds the antigen/MHC complex. Finally, I will discuss the factors that govern repertoire selection. Most of the work discussed will refer to the murine system, which in many aspects is indistinguishable from the human immune system.

T CELL RECEPTOR: MOLECULES & GENES

Clonotypic Antibodies to the T Cell Receptor

Antibody-like molecules, with the ability to confer antigen specificity, were postulated to exist on T cells long before any concrete information about the T cell receptor was available. After the discovery of MHC restriction, T cell receptor models had to account for the dual recognition of antigen and MHC on the surface of APC's. Whether there was one receptor responsible for conferring both specificities, or separate receptors for each, was actively debated (7). In a cell fusion experiment, Kappler et al. (8) showed that specificities for antigen and MHC from the two parent T cell did not assort independently. This argued for the existence of a single T cell receptor, capable of recognizing both antigen and MHC.

T cell receptor structural and functional information was provided by clonotypic or idiotypic antibodies. A number of groups developed clonotypic antibodies in the early 1980's (9-18). These were specific for structures on individual T cell clones or hybridomas. In one example, recognition by a clonotypic antibody successfully predicted antigen/MHC specificity in another independently derived clone (19). Functionally, many of these antibodies could effectively inhibit or stimulate T cell activation depending upon the mode of presentation (16-18).

Immunoprecipitation with the clonotypic antibodies suggested that the T cell receptor was a disulfide linked heterodimer. The two chains were approximately 38-44 Kd each in the mouse while one of

the chains was slightly larger in man. On isoelectric focusing gels, one chain was acidic (called the α chain) and the other was slightly basic (β chain) (10). Two lines of evidence suggested that each chain had variable and constant regions. Peptide maps of TCR's from different T cell clones demonstrated the existence of common and unique peptides (12, 14). Also, while most of the antibodies generated to the T cell receptor were to idiotypic determinants, some were reactive with a broader range of T cells yet still immunoprecipitated the 90 Kd heterodimer (14). A number of proteins, now referred to as the CD3 complex, were shown to be associated with the TCR (20). Comprised of the δ,ε, and γ chains, the CD3 complex participates in signal transduction. Two other chains, ζ and η, have been shown to associate with the TCR (21). The interaction that take place between these proteins and the function of each continues to be explored. Shortly after the wave of structural and functional data from clonotypic antibodies was generated, the genes for the different chains were isolated. Monoclonal antibodies to different T cell receptor determinants remain an invaluable tool for studying T cell biology.

T Cell Receptor Genes

The first T cell receptor gene to be isolated was the β chain gene. Two groups published sequence data simultaneously (22-24). Each had used a different strategy to achieve the goal. Yanagi et al.(22) looked for clones from a T cell cDNA library that were expressed preferentially in T cells. They found 4 clones with similar

restriction maps. The DNA sequence had an open reading frame of 1151 bp's. This predicted a protein with a MW of 35Kd. The authors note a degree of homology to the murine λ light chain and human λ and κ light chains.

Hedrick et al. (23) used subtractive library technology to clone the TCRβ chain gene. This approach was based on the following assumptions about TCR genes: [a] expressed in T but not B cells, [b] mRNA should be in membrane bound polysomes, [c] genes should show evidence of rearrangement, [d] there should be variable and constant regions. They screened a T - B subtracted cDNA library. The probes were labelled cDNA from T cell polysomal RNA that had been subtracted with B cell RNA. Positive clones were used as probes to assess whether DNA rearrangement had occured. One such probe showed evidence of rearrangement. DNA sequences of the original gene isolated plus 3 others picked up from a thymocyte library were compared (14). All had identical 3' regions and different 5' regions. There was a 5' hydrophobic leader sequence and 2 clones shared a 16 residue sequence between V and C regions. It was proposed that this was a putative J region. Homology to the immunoglobulin genes was noted.

The search for the putative α chain was sparked by the successful isolation of the β chain. Saito et al. (25) used the subtractive library technology developed by Davis and coworkers in an attempt to isolate the TCRα chain gene. Two TCR chains were isolated from the CTL clone 2C. One was the β chain and the other, in hindsight, was the γ chain. While this was a gene with many properties of a TCR gene, there were a number of features that

suggested that it was not the α chain gene. There were no N-linked glycosylation sites (where 3 had been found in the α chain of different murine lines). Also, this gene was not expressed in a number of T_h cell lines and levels of expression were lower than expected in the thymus. It would be some time before the partner for the γ chain (the δ chain, discussed below) would be isolated.

The TCRα chain gene, like the β chain gene, was cloned simultaneously by two groups (26, 27). Saito et al. (26) further analyzed the cDNA library from the CTL clone 2C. This led to the isolation of a third TCR gene. This gene had features that made it a better candidate for the α chain. Chien et al. (27) used subtractive library technology too but modified the protocol to select for variable region sequences instead of constant region sequences. A cDNA clone was isolated from a 2B4 (helper T cell hybridoma) library. Sequence analysis, the pattern of expression and evidence of rearrangement suggested that this was the TCRα chain gene. Both published sequences were identical in the C region.

The final TCR gene, the δ gene, was not cloned until 1987. Chien et al. (28) identified a region 85-90 kilobases upstream of Cα that showed systematic rearrangements in adult $CD4^-8^-$ cells and some hybridomas. A rearranged TCR-like gene was isolated from 2B4 within the α chain locus. The C region (originally called Cx) shared many features with Cα. The V region rearranged to this C region had extensive homology to a previously described Vα gene. The function of the γ/δ T cells is still not clear. Their presence in the murine epidermis and intestinal epithelium suggested that γδ T cells may represent a mature T cell population with specific localized

functions. The fact that γδ T cells were predominantly $CD4^-CD8^-$ suggested that interaction with ligand may not be MHC restricted as in the αβ T cells (29).

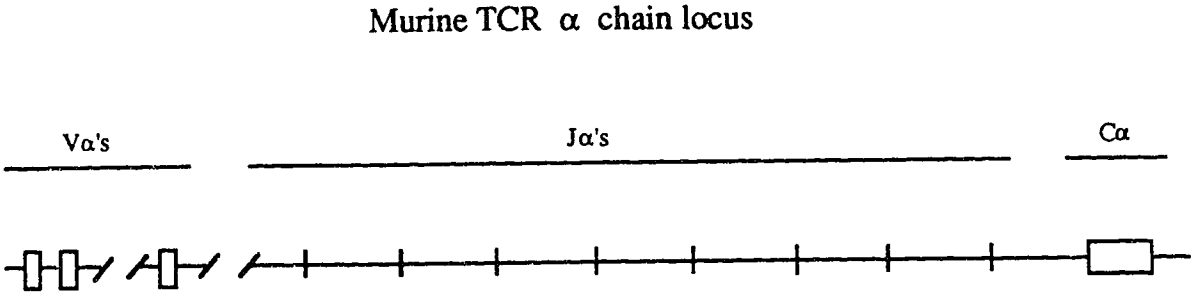
Genomic Organization

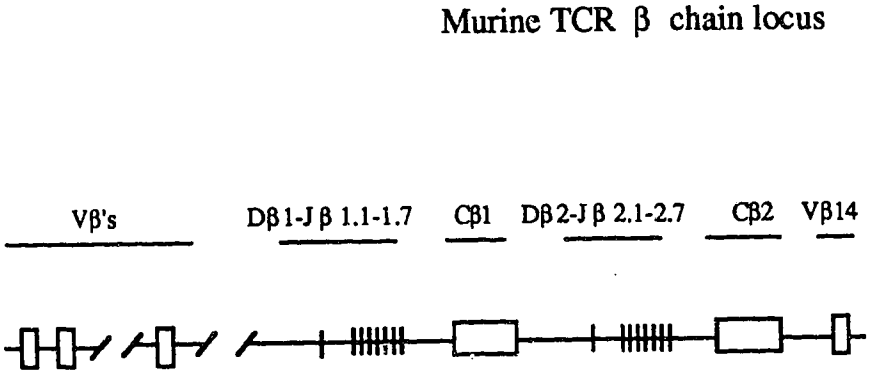
In general, the genes of the 4 TCR chains are all organized in a manner reminiscent of the Ig genes. There are however, unique organizational features that distinguish each gene.

The murine β chain locus is on chromosome 6 (30). It consists of an estimated 30 Vβ genes, followed by 2 D-J-Cβ clusters (31-37). In addition, there is a single Vβ gene (Vβ14) located downstream of Cβ in an opposite orientation (38). Utilization of this Vβ requires an inversion event. Most of the Vβ genes are single member families. Each D-J-Cβ cluster is composed of a single D, 6 functional and one nonfunctional J's, and a single C region. The TCRβ chain locus in the humans is located on chromosome 7 and is organized in a similar fashion (30). There are however more Vβ genes (approximately 50) and one of the J region pseudogenes is functional in humans (39, 40).

The TCRα chain locus in both humans and mice is located on chromosome 14 (41-43). There are 40-50 Vα gene segments (44-49). Unlike the β chain, the Vα genes are grouped into closely related families, each consisting of 4-7 members. No D regions have been identified in the TCRα chain. There are as many as 100 Jα's spread over 70 kilobases between the Vα's and the single Cα region. A schematic representation of the murine TCR α and β chain loci are given in figure 1.1.

Figure 1.1 Genomic organization of the Murine TCR α and β chain loci.





The organization of the murine γ chain locus (chromosome 13) differs from the TCRα, β and human γ chains (29, 42, 50). Distinct clusters of V-J-C regions in different orientations limit the ability to randomly assort gene segments. The human γ chain locus, located on chromosome 7, is organized more like the β chain locus. All the Vγ regions are upstream of 2 J-C clusters (29, 51).

The TCRδ chain locus, while organized in a conventional manner, is unique because of its location. In both humans and mice the δ chain gene is located within the TCRα chain locus, between the Vα's and Jα's (28). There may be some overlap in the V genes used by the δ and α chains (29).

Gene Rearrangement

The rearrangement processes and signals are analogous to those in Ig genes (52, 53). Heptamer-spacer-nonamer signal sequences flank the TCR gene segments. The heptamer and nonamer are conserved sequences that are recognized by proteins involved in the rearrangement event. The spacer regions are either 12 or 23 bp long. As in Ig rearrangement, a 12 bp spacer signal sequence rearranges to a 23 bp spacer signal sequence. The existence of circular excision products, deleted by the rearrangement process, is direct evidence for the looping out model (54-56). Sister chromatid exchange has also been put forward as a model of rearrangement (57). In the case of the murine Vβ14 gene which is in the opposite orientation downstream of Cβ, an inversion event has been shown to occur (38).

Generation of Diversity

There are 3 mechanisms that contribute to diversity in TCR genes. First, is the combinatorial joining of gene segments through gene rearrangement. This is presumed to be a random process whereby any V region can be joined to any downstream J region (or D region where applicable). Secondly, the joining of gene segments is imprecise and N-region diversity exists at the junctions too. N-region diversity is the apparent random insertion of nucleotides at the junctions during rearrangement. Both of these mechanisms result in a great deal of variability in the junction sequences. Finally, the TCR's are made up of 2 chains. Random association of 2 chains (either α/β or γ/δ) increases the number of potential TCR's. These are the same mechanisms used to generate diversity in the Ig genes (58). The somatic hypermutation seen in Ig genes is not found in TCR genes (53). D regions, rearranged in β and δ genes, increase diversity considerably. They can be read in any reading frame and more opportunity for N-region diversification is facilitated by the extra junction.

The α/β TCR has potential for greater diversity than the γ/δ TCR as a result of having more V and J genes to rearrange. Repertoire size for the α/β TCR's has been estimated at 10^7 (59). Actual repertoire size and complexity after thymic selection will be lower. The implications of this for antigen specific responses will be discussed in a subsequent section.

Ontogeny of Expression

The rearrangement and expression of different TCR chains on thymocytes is a developmentally regulated process. In fetal development, γ/δ cells appear first, around day 14 of gestation (60). TCRβ chain rearrangement is thought to occur almost simultaneously with γ and δ (61, 62). However, α/β cells do not appear until after day 16 because α chain rearrangements are delayed (63). It remains controversial whether γ/δ and α/β T cells are derived from separate lineages. In a recent study, the presence of γ/δ transgenes seems to interfere with normal α/β T cell development (64). This indicates some degree of coordination between α/β and γ/δ TCR rearrangement and expression. However, analysis of the circular excision products from TCRα rearrangements did not indicate previous δ chain rearrangements (56). This argues for separate lineages rather than coordinated progressive rearrangements.

Other relevant developmental steps in α/β T cell ontogeny include the relative level of TCR, the association with the CD3 complex during thymic selection and the regulation of other accessory molecules. These topics will be discussed in relation to thymic selection events. The remainder of this review will focus on the α/β T cell receptor and the ability to confer both antigen and MHC specificity.

Transfer of Specificity

With the cloning of the α and β genes of the TCR, there was growing pressure to demonstrate conclusively the functional significance of these gene products. The cloned genes themselves proved to be the key to demonstrating their own function. Transfection of TCR genes into appropriate host T cells followed by functional analysis of the resulting TCR's proved that the α/β chains of the TCR are necessary and sufficient for providing antigen and MHC specificity. The first such study did not transfer antigen/MHC specificity but did reconstitute an active TCR/CD3 complex by transfecting the TCRβ chain into a mutant T cell tumor line (65). Dembic et al. (66) transfered both α and β chain genes from a CTL specific for the hapten fluorescein and H-2D^d. The recipient cell acquired the ability to lyse fluoresceinated H-2D^d targets. This experiment demonstrated that the α and β chains together could transfer specificity. There was no evidence for separate recognition of antigen and MHC. This confirmed earlier T cell fusion studies that suggested that recognition of antigen and MHC determinants was mediated by a single receptor (8, 67). Saito et al. (68) transfered murine TCRα and/or β chain genes into human T cells. Neither chain alone (paired with the other endogenous chain) could confer antigen specificity. Only when both genes were introduced could the donor specificity be demonstrated. Other such experiments helped to confirm the role of the α and β chain genes and give insight into the role of accessory molecules like the CD3 complex, CD4 and CD8 in

antigen recognition (69, 70). Also, the ability of the TCR α/β chains to confer alloreactivity was shown using transfection (71, 72).

The manner in which the TCR recognizes the antigen/MHC complex remains in question today. These transfection experiments showed that a simple universal correlation between antigen or MHC specificity with one or the other chain of the TCR did not exist. Next, numerous well established antigen systems were characterized for TCR gene usage. It was hoped that structure/function correlations would emerge that would provide insight into the rules that govern interactions between TCR and antigen/MHC.

T CELL RECEPTOR: STRUCTURE-FUNCTION CORRELATIONS

Since before the identification and characterization of the TCR, immunologists have attempted to conceptually resolve the intricacies of MHC restricted recognition of antigen by T cells. Evidence that a single TCR was responsible for both antigen and MHC specificity led to the idea of a trimolecular complex of TCR, antigen and MHC (73). Much of our current knowledge of TCR interactions with antigen and MHC has come from the molecular analysis of numerous antigen specific systems. The rationale behind these studies was essentially the same. Working with antigen specific T cell clones or hybridomas whose fine specificity had been characterized, TCR gene usage in these cells was determined. Methodology included the use of TCR V region specific monoclonal antibodies, V region specific DNA probes in Northern analysis, cDNA cloning and sequencing, rearrangement pattern analysis and more recently, the use of the polymerase chain reaction (PCR). Correlations between TCR genes used and the resulting antigen/MHC specificity were sought. Some early reports were inconclusive as a result of small sample sizes and a mixture of fine specificities. As information from more antigen systems was accumulated it became increasingly clear that different patterns of T cell recognition existed for different antigens. For this reason, this discussion of structure/function correlations is organized by antigen system. Numerous types of antigens have been studied, including a variety of peptide antigens, haptens, alloantigens and a relatively new category of antigens called superantigens. A summary table

(Table 1.1) is included to highlight the correlations noted in each antigen system. Each provides information about the T cell recognition of that antigen. Taken together, a complex picture of T cell recognition emerges.

Peptide Antigens

Cytochrome c The first antigen system to be successfully used to show structure/function correlations, the T cell response to pigeon cytochrome c has proved to be the most informative. The predominant T cell response involves the carboxy-terminal 24 amino acids [81-104], restricted on I-E (5). Characterization of the restriction patterns suggested that the I-Eβ chain was the determining factor for antigen - Ia interactions (5). Critical T cell contact residues on cytochrome c mapped to 99(Lys) and 100(Gln) (5). Thus the functional groundwork was in place that made the cytochrome c system appropriate for a molecular approach to T cell specificity.

Fink et al. (74) analysed the TCRα and β gene usage of 4 cytochrome c specific T cell clones by cDNA cloning and sequencing. Analysis of the clones' abilities to respond to moth or pigeon cytochrome c on B10.A ($E\beta^k$), B10.S(9R) ($E\beta^s$), or B10.A(5R) ($E\beta^b$) APC's was assessed. Molecular and functional data from a previously described cytochrome c specific clone, 2B4, was included for comparison. All 5 clones used a member of the Vα11 family (originally named Vα2B4) with one of three different Jα regions. The predominant use of this Vα family was confirmed by Southern

analysis of additional cytochrome c clones from B10.A mice. The TCRβ chain genes used were also limited. Three of the clones used Vβ3 (originally designated Vβ2Bd) with one of 2 Jβ segments. The other 2 clones used Vβ16 (originally named VβB10) rearranged to Jβ2.1. This was the first example of dominant V gene usage in an antigen specific response. While the Vα11 usage was striking, the limited Vβ gene usage could also be considered dominant gene usage. Close examination of the reactivity pattern of each clone and the TCR gene usage allowed the authors to speculate on the importance of a number of TCR regions. For example, acquisition of a new alloreactivity might have been conferred by the use of a different Jα segment. Alteration in antigen reactivity on one APC type might have been caused by junctional diversity differences in the α and/or β chain. Use of Vβ16 (VβB10) may have excluded recognition of cytochrome c on B10.A(5R) APC's. Interestingly, Vβ3-Jβ2.1 which was seen twice in the cytochrome c specific T cell clones was also used in a lysozyme/I-A^b reactive T cell (75). The authors conclude that both the α and β chains were providing components of an antigen binding cleft. The antigen/MHC specificity could be affected by relatively minor changes on either chain.

An extension of this work revealed that the T cell clones could be divided into 4 phenotypic response patterns (76). Looking primarily at rearrangement patterns, the authors note that correlations were apparent between TCR gene segment usage and antigen/MHC specificity. Thus broad response patterns were correlated with gene segment usage. In general, a high degree of receptor selection was observed in both chains of the TCR. The

precise role of junctional diversity was not addressed and a detailed determination of fine specificity using substituted peptide analogues was not done.

The predominance of Vα11 usage in cytochrome c responses was also shown by Winoto et al. (77). Cytochrome c specific T cells from 4 strains of mice were analysed. They compared the T cell receptors from their study with those previously reported and concluded that more than 75% (15/19) of the cells used a member of the Vα11 family. No correlation to MHC restriction patterns could be made. The authors note two interesting pairs of T cells. In one example, two hybridomas differed only by 5 amino acids in the V-D-J junction of the β chain. Fine specificity analysis of these two hybridomas did reveal differences in the response pattern to certain peptide analogues of cytochrome c. Thus while the MHC restriction pattern remained constant, change in junctional amino acids in the β chain did indeed alter antigen fine specificity. In another pair of hybridomas that did not share Vα or Vβ gene usage, no differences in the fine specificity profile was observed. Very different T cell receptors could seemingly create a very similar antigen binding cleft.

Dominant TCR gene usage was also demonstrated in cytochrome c specific T cell clones from B10.S(9R) ($E\beta^s$) mice (78, 79). Interestingly, different Vα and Vβ genes were used. A member of the Vα10 family was rearranged in T cell clones and lines from B10.S(9R). Vβ1 was rearranged to either Jβ1.2 or 2.1 in these cells. The selective pressures that led to this dichotomy of dominant gene usage in these two strains will be discussed later in the context of repertoire selection.

The role of junctional diversity in cytochrome c reactivity was assessed through an extensive sequence analysis (80, 81). Both α and β chains were examined for conserved amino acids in the junctions. No apparent selection for junctional amino acids was seen in the Vα11 sequences. The TCRβ chain on the other hand showed a marked selection for particular amino acids at position 100 (encoded by N or D region nucleotides). In Vβ3 TCR's, an asparagine [N] predominated. In Vβ1 or Vβ16 a aspartic acid [D] was consistently found at position 100. In T cells that were not specific for cytochrome c, yet expressed these Vβ genes, the conserved amino acids were not present. Two cytochrome c specific T cell clones that did not have the conserved amino acids at position 100 had significant differences in fine specificity.

The role of the conserved amino acids at position 100 in cytochrome c responses was tested by site-directed mutagenesis and transfection (82). Two position 100 mutant β chains were generated. Each was transfected alone or with the original TCRα chain. One mutant β chain showed a marked reduction in the ability to respond to pigeon 88-104 and E^k without exogenous antigen, while the response to moth 88-103 was relatively unchanged. The other mutation abrogated all characteristic cytochrome c responsiveness. These studies suggested a critical role for the TCRβ chain junctional sequences in cytochrome c reactivity. This was in addition to the established importance of Vα11. Thus it seems that elements on both chains provide critical components to the antigen binding cleft for this antigen.

The cytochrome c system has also been used to discern the molecular basis of alloreactivity (83). T cell clones with different alloreactivity patterns expressed the same Vα and Vβ gene segments. Rearrangement of unique Jα and D-Jβ segments presumably led to distinct alloreactive specificities. Subtle differences in TCR's either restricted to a particular MHC or alloreactive to it were noted. This suggested that differences in affinity for MHC with and without antigen might exist.

The correlation of MHC restriction pattern with one gene segment or a single TCR chain has been difficult to demonstrate. One study was able to correlate MHC restriction with TCRβ chain (84). Transfection of the TCRβ chain from one cytochrome c specific T cell clone to another resulted in the transfer of the donor restriction pattern. The authors concluded that at least in the donor T cell, the MHC restriction was conferred predominantly by the β chain. Whether this observation can be extended to other T cells remains to be demonstrated.

Myelin Basic Protein (MBP) T cells that respond to MBP have been implicated as causative agents in encephalomyelitis (EAE). EAE is an experimental model for the demyelination disease in humans, multiple sclerosis (MS). T cell clones specific for MBP have been shown to transfer disease (85). The immunodominant region of MBP has been narrowed to the amino-terminal 9 amino acids (p1-9)(86). This portion of the molecule was recognized by the encephalogenic T cell clones. Also, immunization with p1-9 resulted in the induction of

EAE. The restricting element associated with recognition of MBP is I-A^u.

An extensive molecular analysis of the TCR's used by MBP(p1-9) T cell clones has been performed by 2 groups (87-89). As in the cytochrome c response, the T cells involved in the p1-9 response utilized a limited number of α and β chains. The use of Vβ8.2 dominated, accounting for 80-90% of the response. This had clinical relevance in that both groups were able to block EAE with Vβ8 specific monoclonal antibody F23.1. Urban et al. (87) observed the rearrangement of Vβ8.2 only to Jβ2.6, while Acha-Orbea et al. (88) showed rearrangement to Jβ2.3, 2.5 and 2.7. The TCR Vβ's used by the remainder of the T cell clones were either Vβ4 or Vβ13. The TCRα chains used by MBP(p1-9) T cell clones were composed of members of either the Vα2 or Vα4 families rearranged to a limited number of Jα's. The 43 T cell clones analysed by Urban et al. showed a peculiar pattern. Only 2 β chains (Vβ8.2/Jβ2.6 and Vβ13/Jβ2.2) and 2 α chains (Vα2.3/Jα39 and Vα4.2/Jα39) were used but all 4 combinations of α/β were identified. This apparent interchangeability of α and β chains makes correlations to specificity difficult. In a fine specificity analysis of representative clones from each of the 4 receptor types, no differences in fine specificity were detected.

The recognition of MBP(p1-9)/I-A^u utilized a limited repertoire of TCR's. Strong selection for all elements (including junctional sequences) in both chains was evident. This suggested involvement of both α and β chains in conferring specificity.

Sperm Whale Myoglobin T cell clones reactive with the middle CNBr fragment (p56-131) of sperm whale myoglobin were analysed for fine specificity and TCR gene usage (90). Most of the clones isolated were restricted to I-E^d. Fine specificity analysis allowed the categorization of clones into at least 3 reactivity patterns.

Despite the microheterogeneity in fine specificity, most of the clones expressed a member of the Vβ8 gene family. This was assessed using monoclonal antibodies F23.1 (Vβ8.1, 8.2, 8.3) and KJ16 (Vβ8.1, 8.2)(90). Analysis of the junctional sequences using PCR to amplify the region from cDNA demonstrated that Vβ8.2 was being used with either Jβ2.6 (in 5/6 clones) or Jβ2.5 (in 1/6 clones)(91). The TCRα chains used in 6 clones were from 3 different Vα families rearranged to 4 Jα regions. Close comparison of specificity from 3 clones that shared Vα and Vβ genes suggested the importance of junctional and J region sequences in both the α and β chains. In one example, D-Jβ differences were associated with changes in the epitope recognized. In another example, Jα differences were correlated with both alloreactivity and antigen recognition pattern changes. Selection of elements in both chains (albeit more stringently in the β chain) suggested involvement of both chains in myoglobin specificity.

λ Repressor c1 Protein Most of the peptide antigens used in TCR structure function studies were homologous autoantigens from other species. The T cell response to species variant proteins usually was focused around sites of amino acid differences. The T cell response to the first domain of the heterologous bacteriophage λ c1 repressor

protein was characterized (92). In both I-A^d (BALB/c) and I-E^k (A/J) mice, T cell responses were directed to the same immunodominant peptide, p12-26.

The pattern of TCR gene usage by T cells specific for the λ repressor (p12-26) differed slightly from the cytochrome c, myelin basic protein and myoglobin systems. Cytochrome c and MBP responses were dominated by strong selection of all gene segments in both chains. In myoglobin responses, the selective pressure was for elements in the β chain and to a lesser degree, the α chain. The vast majority of I-E^k restricted T cells specific for λ repressor (p12-26) utilized members of the Vα2 family (originally referred to as members of the Vα3 family)(93). While 4 different Vβ genes were associated with Vα2, more than half of the clones used Vβ1/Jβ2.1. Almost the inverse of the myoglobin system, in the λ repressor (p12-26) system the selective pressure was for elements in the α chain and to a lesser degree, the β chain. Subsequent analysis of the junctional diversity associated with the dominant V genes showed very limited diversity in the Vα2 chains (94). While 7 of the 8 clones used Jβ2.1 with Vβ1, 4 different junction sequences were identified. Conservation of a glutamic acid residue at position 100 in these junctions, similar to the cytochrome c system, suggested a possible role in conferring specificity.

Lysozyme The TCR of 7 T cell hybridomas specific for hen egg lysozyme use 5 Vα and 5 Vβ genes (95). Partial dominant gene usage was evident in both chains. In 7 hybridomas analysed, 3/7 used Vβ14 and 3/7 used a new Vα designated VαC10. The panel of

hybridomas was a mixture of antigen fine specificities and MHC restriction patterns, making structure/ function correlations difficult.

Insulin The T cell receptor V genes used in the I-A^b or I-A^{bm12} restricted insulin responses were diverse (96, 97) No structure/function correlations were readily apparent. Rigorous fine specificity analysis is difficult with insulin due to structural features of the T cell epitope on this molecule (98).

Hapten Antigens

A number of hapten systems have been characterized for structure/function correlations too. It was hoped that the small size of the antigenic moiety might elicit a narrow T cell response. In 3 hapten systems examined, three different TCR gene usage patterns were observed. The range of response patterns seen in peptide antigens was paralleled in the hapten systems.

TNP/H-2K^b Cytotoxic T cell (CTL) clones specific for 2,4,6-trinitrophenyl (TNP)/H-2K^b predominantly used one TCR (99, 100). In 42 independent T cell clones, 16 (40%) used TCR's composed of identical V-Jα, V-D-Jβ elements and junctional sequences. This argued for strong selective pressure for all components of the TCR to generate TNP/H-2K^b reactivity. It is important to note that these T cell clones were derived from a number of lines, otherwise this pattern of TCR gene use could arise from one clone dominating the culture prior to cloning.

pABA/I-A^d or I-A^k Tan et al. (101) characterized the TCR's used in p-azobenzenearsonate (pABA) specific T cell responses. They showed strong selection for Vα3 rearranged to 2 or more Jα segments. At least 3 different Vβ genes were associated with Vα3. A number of lines of evidence suggested that Vα3 was largely responsible for conferring reactivity to pABA in association with 3 different MHC proteins.

AED/K^b or D^b Molecular analysis of 4 *N*-iodoacetyl-sulfonic-naphthyl-ethylene-diamine (AED) specific T cell clones was unable to detect any predominant V gene usage (102). More clones would be needed in each specificity group to draw structure/function conclusions from this system. This preliminary work suggested that strong selective pressure for certain TCR's, as seen in the TNP and pABA systems did not exist.

Alloantigens

The molecular basis of alloreactivity has been examined from two perspectives: as a crossreactivity in antigen specific systems (discussed in the cytochrome c section) and in T cells raised specifically against allodeterminants. The role of peptides that may be associated with allo-MHC in these responses is not known. The high precursor frequency of T cells that can recognize alloantigens distinguishes this type of recognition from nominal antigen systems. A number of studies have used the T cell response to the H-2^b bm

series of mutants to look at TCR gene usage. These mutants differ from the parent molecules by as few as 3 amino acids and therefore represent a defined epitope.

H-2K^{bm1} and D^{bm14} The Vβ8 gene usage in the H-2K^{bm1} and D^{bm14} responses was determined using monoclonal antibody F23.1 (103). T cells from individual mice were assessed. A consistent and significant fraction of T cells in both responses used a member of the Vβ8 family (36% in K^{bm1} and 45% in D^{bm14}). While this represented a predominant portion of the response, the number of other Vβ's and α chains involved in the response could be very large. As this was not addressed by the study it is difficult to make inferences about the T cell repertoire used in this system.

I-A^{bm12} A rigorous look at TCR V gene usage in 178 I-A^{bm12} reactive T cell hybridomas was performed by Bill et al. (104). The I-Aβ gene of bm12 differs from I-Aβ^{b} by 3 nucleotides which give rise to 3 amino acid changes in the first extracellular domain of the molecule. Using V region specific probes in a dot blot analysis, hybridomas were characterized for Vα and Vβ usage. The frequency of V gene usage in these I-A^{bm12} specific hybridomas was compared to unselected hybridomas from the same strain. Using 11 Vα and 16 Vβ probes, 9 Vα and 13 Vβ genes were detected in the panel. No significant shifts in Vα gene usage were detected. Overexpression of Vβ14, 15, and 16 and underexpression of Vβ5 was noted. Junctional diversity in a number of hybridomas was assessed. No conserved

junctional sequences or J regions could be correlated with I-A^{bm12} reactivity.

At least 37 (and up to 67) different TCR's were estimated to participate in the I-A^{bm12} response. The size of the repertoire might appear remarkable if that all of these TCR's were specific for only the 3 amino acids that distinguish I-A^{b} from I-A^{bm12}. However, the T cells had been positively selected for moderate I-A^{b} reactivity during thymic maturation. The fact that so many different TCR's were associated with I-A^{bm12} reactivity might reflect the inherent flexibility of TCR recognition. The I-A^{bm12} system was ideal to demonstrate this. Hypothetically, the subtle increase in affinity could be conferred by almost any element of the TCR if a permissive set of other elements existed. Certain Vβ regions may have been responsible for conferring reactivity but only when paired with particular α chains or in conjunction with permissive D-Jβ sequences. Other Jα's may have had the same effect but only when rearranged to certain Vα's or paired with some β chains. Some V genes might have inhibited interactions, thereby reducing the likeliness of being detected in the response. Thus the apparent randomness in gene usage with exceptions that are more or less frequent than expected.

Superantigens

The term superantigen has emerged to describe a group of antigens that interact with the TCR in a distinct manner. T cell recognition of these antigens is correlated with the expression of certain Vβ genes. Reactivity, is therefore thought to be conferred by

elements in the Vβ region alone. The role of D-Jβ and the α chain in these responses is greatly diminished. As a consequence of this, almost all T cells bearing a particular Vβ gene will respond to certain superantigens. During thymic development, the presence of a superantigen will lead to the near complete deletion of thymocytes bearing particular Vβ genes. These systems have been used effectively to demonstrate positive and negative selection in the thymus. This aspect of these systems will be discussed in relation to repertoire selection in a subsequent section of this review. This section will concentrate on superantigen interaction with TCR and how this relates to TCR function.

Unknown self Ag + I-E Recognition of I-E class II MHC molecules has been correlated with expression of Vβ17a (105). Whether recognition involves a particular self peptide in the groove of the I-E molecule itself has not been clarified. The correlation to I-E reactivity has been shown by a number of criteria. The majority of randomly selected hybridomas bearing Vβ17a reacted to splenocytes that expressed I-E. At least 8 Vα genes were expressed by these hybridomas, indicating that α chain involvement in I-E reactivity was unlikely. Also, strains that expressed I-E had a marked reduction in peripheral Vβ17a T cells (106). This indicated that expression of Vβ17a allowed contact with I-E in the thymus and led to the subsequent deletion of those cells. From the standpoint of antige recognition the strength of this correlation was unprecedented.

Staphylococcal Enterotoxins (SE's) The apparent mitogenic effect of SEB on T cells was shown to require class II MHC. This fact and the observation that T cell reactivity to SE's was clonally expressed, suggested an involvement of the TCR in the response (107). The TCR's that conferred reactivity to SEB used either Vβ3 or any member of the Vβ8 gene family (108). Almost all T cells bearing these Vβ genes were stimulated by SEB. Neonatal tolerance to SEB resulted in almost complete deletion of these Vβ genes from the peripheral repertoire. A panel of Staphylococcal enterotoxins tested stimulated T cells bearing certain Vβ genes also (109). Each of the nine toxins stimulated a distinct set of TCR Vβ genes. All toxins possessed superantigen properties. Some individual T cells did not fit the predicted response patterns. In these examples, involvement of non-Vβ gene segments in conferring reactivity to the SE's was postulated. Direct binding of intact SEA and SEB to HLA-DR proteins (human class II MHC) has been demonstrated (110). Also, a potential interaction site on one Vβ has been identified (111). Based on TCR models these residues were predicted to lie outside the antigen binding region. This would account for the apparent exclusive association with Vβ regions regardless of the other elements of the TCR.

Minor Stimulatory Antigens (Mls) The minor stimulatory antigens (Mls) locus was discovered as a non-H-2 locus capable of inducing strong mixed lymphocyte reactions (MLR's) (112). There are no serologic reagents available to define Mls alleles. Only T cell clones can identify Mls products. Early work by Festenstein suggested a

single locus with 4 alleles, $Mls^{a,b,c,d}$ (112). Closer examination of the system revealed that there were in fact only 2 alleles, Mls^a and Mls^c, with Mls^b a null allele (expressing neither) and Mls^d a mixture of both (113). Segregation analysis concluded that Mls^a and Mls^c were distinct and unlinked genes (114). Due to the genomic arrangement of the Mls genes, Mls^a and Mls^c are now commonly referred to as Mls-1^a and Mls-2^a respectively, with Mls-1^b and Mls-2^b as the null alleles.

The enormous T cell response elicited by Mls-1^a was shown to involve nearly all T cells bearing either Vβ8.1 (115) or Vβ6 (116-118). In both cases, a large fraction of randomly chosen T cell hybridomas or clones using TCR's with appropriate Vβ regions were stimulated by Mls-1^a. Also, those strains that expressed Mls-1^a deleted T cells in the thymus that used these Vβ genes. Subsequently, another Vβ gene (Vβ9), was correlated with Mls-1^a reactivity (119). Potential interaction sites on Vβ8.1 have been identified in a mutagenesis study (120). Interestingly, one site at amino acids 70/71, corresponded to the region identified in the SEB-TCR interaction.

Recognition of Mls-2^a has been correlated with expression of Vβ3 by 2 groups (121, 122). Both studies showed reactivity of $Vβ3^+$ hybridomas to Mls-2^a splenocytes while hybridomas expressing other Vβ's did not respond. Also, strains that were Mls-2^a deleted thymocytes expressing Vβ3.

The primary determinant of superantigen specificity is clearly certain Vβ genes. The strong correlation of superantigen recognition with only Vβ genes led to speculation that superantigens bound the

TCR outside the conventional antigen binding cleft (123). This premise is supported by TCR mutagenesis studies in SEB and Mls-1a binding (111, 120). Interaction sites identified in both systems are potentially outside the antigen binding cleft. The role of D-Jβ and the α chain appears to be secondary. However, there are examples of TCR's that express a given Vβ gene yet do not respond to the appropriate superantigen (109, 116 and this work). Also, it is very difficult to show complete deletion of a subpopulations of T cells with MAb's in a FACS analysis. The exceptions to the strong correlation to Vβ genes may be a result of the influence of other components of the TCR. If the superantigens only bind TCR outside the antigen binding cleft, then conformational changes may account for some exceptions. On the other hand, part of the interaction with superantigen may involve other elements in the cleft. The exact manner in which TCR binds superantigens is not known. The strong correlation of superantigen recognition to Vβ genes may represent one extreme type of antigen recognition. Other antigen systems provide examples of the range of antigen recognition patterns. These patterns range from an apparent strict requirement for all elements in both chains before antigen reactivity is achieved, to a predominance of one element or one chain in determining reactivity. A summary table of the dominant V gene usage in the antigen systems discussed is included (Table 1.1).

This thesis is based on the T cell response to a synthetic peptide antigen, poly-18. The pattern of reactivity to this antigen will be discussed in relation to the other antigen reactivity patterns reviewed here.

Table 1.1

T Cell Receptor Structure-Function Correlations

Antigen	MHC Restriction	Dominant TCR Usage Vα	Vβ	Reference
Peptide				
Cytochrome c	I-E^k	11	3	74,76,77
(81-104)	I-E^s	10	1	78,79
Myelin Basic Protein (1-9)	I-A^u	2, 4	8.2, 13	87-89
Sperm whale Myoglobin (110-121)	I-E^d	Mix	8.2	90,91
λ Repressor c1 Protein (12-26)	I-E^k, I-A^d	2	1	93,94
Hen Egg Lysozyme (34-45 or 13-105)	I-A^k	C10+/-	14+/-	95
Insulin	H-$2^{b,bm12}$	Mix	Mix	96,97
Hapten				
TNP	K^b	4	3	99,100
AED	K^b, D^b	Mix	Mix	102
pABA	I-A^d, I-A^k	3	Mix	101
Alloantigen				
-	K^{bm1}, D^{bm14}	ND	8+/-	103
-	I-A^{bm12}	Mix	Mix*	104
Superantigen				
Unknown Self Ag	I-E	Mix	17a	105,106
Mls-1^a (Mls^a)	-	Mix	6, 8.1, 9	115-119
Mls-2^a (Mls^c)	-	Mix	3	121,122
SEB	-	Mix	3, 8.1-8.3	108,109

* Authors note an enrichment for Vβ14, 15 & 16.

+/- Dominant V gene usage not shown conclusively.

Structural Models

The conceptual framework of the trimolecular complex was proposed before any structural information existed about the elements involved (73). Inherent in this model was the complex array of interactions that would exist between the 3 molecules involved. Antigen-MHC, antigen-TCR and TCR-MHC contact sites were proposed. Evidence has been accumulated in support of all three of these types of interactions.

Crystallographic structure of the human class I histocompatibility antigen, HLA-A2, was determined by Bjorkman et al. (124). The structure of class I differed from immunoglobulin C and V regions. The α_1 and α_2 domains combine to from a β pleated sheet platform with 2 α-helices on top. The groove created by the two α-helices may be the peptide binding site proposed by peptide binding studies and competition experiments (125). The placement of the amino acids in HLA-A2, as determined from the crystallographic data, was consistent with previously identified residues accessible to antibodies or critical for contact with antigen or the TCR (125). It was suggested that class II MHC molecules will share structural features with class I MHC (126).

Current models of antigen recognition by the TCR are based largely on the structure of the HLA-A2 molecule, the assumption that the 2 α-helices form the peptide binding groove and that the TCR will have structural similarities to antibody molecules. Extensive amino acid sequence analysis of TCR α and β chains and Ig H and L chains strongly suggested the existence of conformational similarities between TCR and Ig molecules (127). There was limited sequence

variation in the first and second hypervariable regions, also referred to as complementarity-determining regions (CDR's). These are encoded by Vα and Vβ genes. The third CDR, encoded by the V-D-Jβ and V-Jα junctions had the greatest degree of variability. In antibodies, the first and second CDR's from each chain flank the third CDR's (128). Davis and Bjorkman (129) and Chothia et al. (127) suggest that the first and second CDR's may contact the two α-helices of the MHC molecule while the third CDR from each chain contact the antigen in the groove. The spatial arrangement of chains would allow for such interactions. The authors suggest that simple correlations with TCR V regions that would support such a model may not be readily apparent. A number of recognition sites along the MHC α-helices may exist. This combined with a degree of flexibilit in how peptides are positioned in the groove make the subtleties of T cell recognition complex to analyse.

T CELL RECEPTOR: REPERTOIRE SELECTION

An antigen specific T cell receptor repertoire represents a small subset of the total peripheral T cell receptor pool. The composition of an antigen specific T cell repertoire is determined by a number of interconnected factors. Thymic selection events associated with T cell maturation determine the size and complexity of the peripheral T cell repertoire. The MHC haplotype and a number of other genes play a pivotal role in this process. An enormous amount of work has been done in an attempt to understand the process of T cell maturation that takes place in the thymus. In the periphery, antigen acts on the available T cell repertoire to activate a small subset of T cells that recognize immunodominant regions of the antigen in association with products of the MHC. In the context of an antigen specific repertoire, T cell selection events associated with maturation as well as determinant selection, both play a major role in T cell receptor repertoire selection. This review will concentrate on the more recent advances that have clarified some of the issues in these fields considerably. Emphasis will be placed on the consequences of thymic selection events and determinant selection as they pertain to shaping the peripheral T cell repertoire.

Thymic Selection

Thymic selection must account for 2 properties of mature T cells: firstly, T cells recognize antigen in an MHC restricted manner and secondly, they are tolerant to self antigens. The shaping of T cell

recognition in the thymus was originally termed adaptive differentiation. Classic experiments involving $(P_1 \times P_2)F_1 \rightarrow P_1$ or P_2 radiation chimeras demonstrated that the genotype of the thymus determined the restriction specificity of the emerging T cells (130-133). Experiments involving thymectomized mice, followed by replacement with a thymus from another haplotype, led to the same conclusions (134,135). Namely, that the restriction specificity of mature T cells was not genetically determined by the T cell, but rather was imposed or selected for in the thymus. This appeared to be true for both class I and class II restricted T cells. Which cells in the thymus were responsible for imposing these properties on incoming thymocytes? Two candidates were readily apparent: epithelial cells as part of the thymic stroma and/or bone marrow derived macrophage or dendritic cells. The process of skewing T cell recognition toward self MHC was thought to involve either negative selection (136) or positive selection (137). As we will see later in the review, evidence for both of these selection schemes has been generated.

The current view of T cell development suggests that immature thymocytes rearrange and express the αβ TCR on their surface. The ability of a given TCRαβ combination to interact with self-MHC in the thymus seems to determine whether the thymocyte will live or die. It has been known for some time that the vast majority of thymocytes die in the thymus, while only a small proportion go on to exit the thymus and make up the peripheral repertoire. There are a number of steps that can lead to cell death. Failure to successfully rearrange and express a functional TCR presumably leads to cell

death. Strong interactions with self-MHC molecules leads to clonal deletion or negative selection. An inability to interact with self-MHC, ie. a failure to be positively selected, also leads to cell death. The cells of the thymus that are responsible for positive and negative selection steps may be different. Negative selection, leading to a state of self tolerance, is thought to involve interaction with MHC on bone marrow derived dendritic cells or macrophages. Positive selection, resulting in the self-MHC restricted recognition pattern, is thought to involve interactions with MHC on the thymic epithelium itself.

Thymic Selection: Negative

Negative selection or clonal deletion of autoreactive thymocytes has been clearly demonstrated using the superantigen systems discussed previously. The correlation of I-E recognition with Vβ17a was the first system in which clonal deletion could be demonstrated (106). T cells bearing Vβ17a and therefore specific for a self antigen on I-E were deleted during thymic maturation in strains that expressed I-E but were present in strains that did not. Vβ17a was shown to be expressed in the thymus yet absent from the periphery. Essentially identical results were obtained from the other superantigen systems. Expression of Mls-1^a was associated with the near absence of Vβ6, Vβ8.1 and Vβ9 expressing T cells from the periphery of mouse strains that expressed it (115,116,119). Interestingly, some examples of T cells that were unable to recognize Mls-1^a despite their expression of these Vβ genes were noted (116).

While not detracting from the clear demonstration of negative selection in the thymus, these exceptions become important in models of T cell receptor interactions with superantigens. Neonatal tolerance was shown to be mediated by a clonal deletion mechanism also (108,117).

TCR transgenic mice were also used to illustrate negative selection. These mice expressed functionally rearranged TCR genes of a given specificity. Depending on the TCR gene used and the strain of mouse that the gene was expressed in, thymic deletion of T cells bearing the transgene was predicted. For example, double transgenic mice were generated that expressed both α and β chains of a TCR specific for the male antigen H-Y on H-2D^b (138,139). In male H-2^b mice, where the H-Y antigen was expressed, few T cells expressing the transgene matured beyond the double positive (CD4+, CD8+) stage. The females on the other hand, showed no evidence of negative selection, generating large numbers of CD8$^+$ mature T cells. In another system, TCR transgenic mice that expressed a Vβ3 chain deleted thymocytes in Mls-2^a strains (140).

A number of negative selection studies suggest that the selection step occurs prior to complete maturation, most likely at the double positive stage. Histological staining of thymocytes from Mls-1^a mice, which delete Vβ6 suggested that Vβ6 cells were deleted at the corticomedullary junction (116). Kappler and coworkers came to the same conclusion in both the Vβ17a/I-E and the Vβ8.1/Mls-1^a systems (105, 106). In the Vβ3 transgenic mice that also expressed an α chain transgene, rapid deletion of most of the CD4+ CD8+ double positive population was observed (140). The authors speculated that

the presence of both transgenes may have sped up the developmental process and led to deletion at an earlier stage than in normal thymocytes. Fowlkes et al. blocked the deletion of Vβ17a thymocytes in I-E$^+$ strains through *in vivo* treatment with monoclonal antibody specific for CD4 (141). Since both CD4 and CD8 Vβ17a$^+$ T cells were found in the periphery, the authors concluded that negative selection must normally occur at the double positive stage of thymocyte development.

Most of the early work on T cell maturation suggested that negative selection was mediated primarily by bone marrow derived cells in the thymus (reviewed in 6). Radiation chimeras between I-E$^+$ and I-E$^-$ strains of mice were used to show that the deletion of Vβ17a could be mediated by I-E$^+$ bone marrow derived cells (142). While the bone marrow derived cells in the thymus may be responsible for negative selection, some studies suggest that the ability to facilitate deletion may not be an intrinsic feature of the APC (143). Rather, the developmental stage of the thymocyte at the time of contact could be the critical determinant of the fate of the thymocyte. Interactions between TCR and the CD3 complex have been implicated as important in this process (144, 145). Two populations of TCR$^+$ thymocytes were shown to exist, one in which the TCR-CD3 were coupled to each other and the other where they were not (144). The signal transduced by each complex differed quantitatively (145). The physiological implications of this difference remains unknown.

The involvement of TCR in the negative selection step has been well documented in recent years. Stimulation of immature

thymocytes or T cell hybridomas with monoclonal antibodies to the TCR, *in vitro* and *in vivo* led to cell death by apoptosis (146, 147). The signal delivered to these cells resulted in cell death whereas other more mature T cells would be activated by the same signal. This further argued that the developmental stage of the thymocyte at the time of interaction with ligand in the thymus dictates the fate of the cell.

The process of negative selection has direct consequences for the size and complexity of the peripheral TCR repertoire. Certain TCR $\alpha\beta$ combinations are clearly being deleted. In the case of superantigen related deletion, a significant proportion of the potential T cell receptor repertoire is deleted. The question remains whether this negative selection step deletes a broad enough range of T cells to adversely affect the immune system's ability to mount an effective immune response against an invading pathogen. Vidovic and Matzinger describe an example where tolerance to a self antigen causes unresponsiveness to the synthetic antigen GT (148). However, given the complexity of most T cell responses to even relatively small antigens, this type of unresponsiveness as a result of clonal deletion will probably be rare.

Thymic Selection: Positive

Weak interactions between thymocyte TCR and MHC molecules on the thymic epithelium are thought to be the critical events in

positive selection. The bias of the resulting T cell receptor repertoire toward recognition of self-MHC leads to the MHC restricted pattern of recognition seen in peripheral T cell. Those thymocytes that are not positively selected, presumably undergo a programmed cell death. The identification and characterization of the αβ TCR as the receptor for both antigen and MHC effectively eliminated the two receptor model of T cell recognition. This meant that the selection of T cells capable of recognizing antigen and MHC had to operate within the framework of the one receptor (with dual specificity) model. Recent years have seen the accumulation of direct and indirect evidence for positive selection of thymocytes on the basis of the TCR expressed. Much of this evidence comes from the use of TCR and MHC transgenic mice and TCR V region specific monoclonal antibodies to follow the fate of certain thymocytes.

Kruisbeek and coworkers treated murine neonates with MAb's specific for either class I or class II MHC antigens (149, 150). This effectively inhibited the development of mature CD8+ and CD4+ T cells respectively. This was interpreted as evidence that positive selection of both class I (CD8+) and class II (CD4+) restricted T cells required interaction of TCR with MHC molecules in the thymus.

A number of other systems described recently supported the notion that contact between TCR and MHC facilitates positive selection. Transgenic mice expressing TCR αβ specific for the male antigen H-Y in association with H-2D^b were generated. An elevated number of mature CD8+ T cells were observed in transgenic females only when the maturation took place in an H-2^b thymus (151). Bone marrow from these transgenic mice used in chimera experiments

showed that the increase in peripheral CD8+ T cells only occured in strains that expressed D^b (152). Introduction of the SCID mutation into these TCR transgenic mice confirmed the requirement for the presence of D^b in the thymus before positive selection could occur (153). Another class I restricted TCR αβ transgenic system followed a similar developmental pathway (154, 155). TCR αβ genes from a T cell clone derived from BALB.B (H-2^b) and specific for the class I molecule L^d were used to generate the transgenic mice. Thymic development in H-2^b mice generated a predominance of CD8+ mature T cells (154). The same transgenes did not generate large numbers of CD8+ T cells in an H-2^s strain (155). These experiments illustrate the need for the presence of the class I restricting element in the thymus so that positive selection could occur. The apparent absence of positive selection in strains that expressed an inappropriate MHC resulted in the inability of thymocytes expressing these TCR genes to develop to maturity.

Similarly, transgenic mice expressing TCR αβ genes that conferred specificity for a fragment of cytochrome c plus I-E^k were used to illustrate positive selection of class II restricted T cells (156, 157). Kaye et al. showed a developmental bias toward CD4+ T cells in mice that expressed I-E^k (156). Interaction with the I-E^k element in the thymus was proposed to explain the developmental bias. Berg et al. also showed that development of thymocytes bearing the transgenes were augmented in H-2^k mice (157). This was in contrast to H-2^b transgenic mice where the positive selecting element is absent. These mice show arrested development in the thymus at the double positive stage. Expression of an I-Eα transgene on cortical

epithelial cells in the thymus of H-2^b mice allowed the development of the TCR αβ transgene expressing thymocytes.

In nontransgenic systems, positive selection of some Vβ genes has been correlated with the presence of certain MHC molecules in the thymus. In Mls-1^b strains, which do not delete Vβ6 thymocytes, the presence of I-E had a positive influence on the frequency of CD4+ Vβ6+ mature T cells (158). Certain alleles of I-E had more effect on the Vβ6 frequency than others. Evidence for the positive selection of Vβ17a+ thymocytes by H-2K^s molecules has been generated (159). The frequency of mature CD8+ Vβ17a+ T cells was dramatically reduced by *in vivo* treatment with anti-K^s MAb while anti-D^s had no effect.

The crystallographic structure of class I MHC molecules suggests that the peptide binding groove may normally be occupied by self peptides. Nikolic-Zugic and Bevan have attempted to assess the role of self peptides in the positive selection of T cells able to recognize an ovalbumin (OVA) peptide plus H-2K^b (160). Using a series of K^{bm} mutants, they correlated the ability to effectively present the OVA peptide with the generation of mature T cells able to recognize the OVA peptide plus H-2K^b. They suggest that self peptides that mimic OVA were responsible for positively selecting T cells that would later be able to bind OVA. The inability to present these "OVA-like" self peptides by some of the mutants resulted in no positive selection for T cells that could respond to OVA, hence the hole in the repertoire. This is the only work that has attempted to demonstrate a role for self peptides in positive selection. In the

same way that MHC molecules can be involved in both positive and negative selection, self peptides may have a role in each process too.

There is some disagreement regarding the developmental stage at which positive selection takes place. Most of the systems suggest that positive selection occurs at the double positive (CD4+,CD8+) stage of thymocyte development. However, I-E transgenic mice that express I-E on different cells of the thymic epithelium or of bone marrow origin have been used to assess when and where positive selection occurs (161). Changes in the frequency of Vβ6 T cells as a result of I-E expression was used as a gauge of positive selection. Thymocytes from I-E+ transgenic mice and I-E- littermates were evaluated for CD4, CD8 and TCR Vβ6 expression. The only subpopulation that showed evidence of positive selection was the CD4+8-, $V\beta6^{hi}$ group. Thus the authors concluded that positive selection must have occured rather late in thymocyte development, after the switch to single positive phenotype. This is in contrast to TCR transgenic mice that were expressed in strains that did not positively select for the transgenes, resulting in a block at the $CD4+8+TCR^{lo}$ stage of development (157). The possibility that the switch to single positive phenotype and positive selection may be tightly connected events could not be ruled out in these experiments. Whether positive selection precedes negative selection or follows it may depend on the nature of the element being negatively selected. Hengartner, Zinkernagel and coworkers describe a system whereby tolerance to a classical MHC restricted antigen occured prior to positive selection while negative selection for Mls-1a reactivity occured after positive selection (162, 163).

The panel of I-E transgenic mice described above were used to assess which cells were responsible for positive selection of Vβ6 thymocytes (161). This study and a number of others all agreed that the selecting element must be on epithelial cells of the thymic cortex for positive selection to occur (157, 161, 164).

In each example where positive selection was demonstrated, the CD4 and CD8 phenotype of the resulting T cells correlated with the MHC molecule involved. If class I molecules were implicated in the positive selection, the resulting T cells were skewed toward CD8+ (150-155, 162, 163). Likewise, if class II molecules were involved, then the T cells generated were CD4+ (149, 156, 157). This association between CD4,CD8 phenotype and the restriction specificity of the T cells has been known for some time. These molecules are thought to interact with constant portions of the class I and II MHC molecules, potentially stabilizing TCR interactions with Ag and MHC (165). Their apparent participation in the positive selection step explains the origin of this association. It has been suggested that the divergence of CD4+ (helper) and CD8+ (cytotoxic) functional phenotypes may in fact be initiated by signals from CD4 and CD8 at the time of positive selection (166).

The existence of a positive selection step in the thymic maturation of T cells has now been documented in a number of systems. This step clearly affects the composition of the peripheral T cell receptor repertoire. MHC molecules play a central role in this process yet other non-MHC genes and self peptides seem to be involved as well. This is also true of negative selection. Together, positive and negative selection determine the peripheral T cell

repertoire. While it hasn't been assessed directly, this implies that the T cell repertoire is determined by the genotype of the individual. The MHC and TCR loci represent only part of the multifactorial determination of repertoire size and complexity. The generation of an antigen specific T cell response from the available peripheral T cell repertoire superimposes another level of complexity on the system. The antigen will be processed and presented to the T cells in an MHC restricted manner by antigen presenting cells. This process is also controlled by a number of genetic factors. Termed determinant selection, this process further shapes the composition of an antigen specific T cell repertoire.

Determinant Selection

Characterization of the phenomenon of MHC restricted T cell recognition led to the discovery that the antigen presenting cell presented only a select few antigenic fragments to responding T cells. Rosenthal and coworkers proposed a model of determinant selection to explain Ir gene control of immune responsiveness to insulin in guinea pigs (167). Heteroclitic T cell response patterns to pigeon and moth cytochrome c with different APC's supported the idea that Ag-Ia interactions were necessary for T cell responses (5). From these studies, the existence of a trimolecular complex of TCR-Ag-MHC was proposed. Study of immunodominant regions of proteins led to the suggestion that certain structural features of the antigen were required for interaction with MHC (168). Experiments that used related and unrelated peptides to compete for Ia binding sites

suggested that a limited number of peptide binding sites existed (169, 170). Actual interactions between antigenic fragments and Ia molecules were eventually shown to exist, lending support to the models of determinant selection and the trimolecular complex (171-173). Detailed fine specificity analysis using substituted peptides allowed the identification of distinct TCR and MHC contact residues on the antigen (174, 175). The ability of a given Ia molecule to bind a peptide antigen was correlated with the ability to raise a T cell response to that antigen in that strain (176).

Endogenously synthesized proteins are primarily restricted to class I MHC while antigens taken in from outside the cell interact primarily with class II MHC. This dichotomy of restriction specificities may be related to how the molecules are transported to the cell membrane. Teyton et al. (177) have shown that the invariant chain associates with class II MHC in the endoplasmic reticulum (ER), thereby preventing interaction with endogenously synthesized peptides. Class I MHC on the other hand, are thought to associate with peptides while still in the ER. Class I association with peptide may even be required for transport to the membrane.

A modified version of the determinant selection hypothesis, called determinant protection, suggested that immunodominant regions of antigen were bound by Ia while still in the lysosomal vesicle (178). This would protect these regions from further degradation, allowing them to be presented to the T cells. Experimental evidence exists to support this model (179).

Based on the crystallographic structure of MHC class I, a peptide binding site exists in the groove created by the two α-helices

on the β-pleated sheets (124, 125). Sequence comparison between class I and II proteins has led to speculation that a similar antigen binding cleft would exist on class II MHC as well (126). Comparison of antigenic fragments that can be bound by the same MHC molecule suggested that common amino acid motifs might account for the ability to be presented (107, 175).

Immunodominance created by selective degradation and association with MHC molecules dictates which antigenic fragments are presented to T cells. This competition for MHC molecules will be associated with antigenic strength. Determinant selection by the APC, in conjunction with the antigen specific T cell precursor frequency, are the major factors that determine the magnitude of a T cell response. Our knowledge of T cell recognition still does not allow us to predict antigen strength. Comprehension of the factors that govern T cell repertoire generation in the thymus is only now unlocking the molecular mechanisms that underlie the selection steps. Detailed information about all aspects of T cell biology are necessary for fields like vaccine development, organ transplantation and the study of autoimmune disorders.

PROJECT AND RATIONALE

An enormous amount of information on the T cell receptor has been generated in the six years since the first genes were cloned. General rules governing T cell recognition of Ag-MHC have not emerged. Rather, T cell receptor gene usage in at least 20 antigen systems has provided insight into the range of response patterns that exist.

This thesis concerns the molecular analysis of the T cell response to poly-18. Poly-18 is a synthetic peptide antigen composed of the sequence, $[EYK(EYA)_5]_n$. The antigen was designed to contain a limited number of amino acids in a defined order and in an α helical conformation (180, 181). The T cell response to poly-18 is under Ir gene control. H-2^d mice (BALB/c and DBA/2) are high responders while H-2^b (C57BL/10J) and H-2^k (C3H.HeJ) are nonresponders (182, 183). Broad functional analysis of T cell responses to this antigen reveal 2 response patterns: one requiring the lysine [K] residue and the other not (184). Closer examination of the response patterns within each group reveals a high degree of microheterogeneity (185). Thus the apparently simple composition of poly-18 does not appear to elicit a simple spectrum of reactive T cells. Molecular analysis of the T cell receptors involved in the poly-18 response offers the opportunity to correlate functional characteristics of the system with T cell receptor gene usage. Analysis of the poly-18 system is also meant to complement the work done in other antigen systems. Together, a picture of T cell

recognition can emerge that more accurately reflects the array of T cell response patterns that exist.

The work on the T cell receptor repertoire to poly-18 focuses on the T cell response in two strains of mice. The first two studies focus on the poly-18 response in BALB/c mice. Analysis of a panel of poly-18 reactive T cell hybridomas led to a profile of TCR gene usage in this response (Chapter 2). Specific objectives of this study are:

1. Characterize TCR Vα and β gene usage in a panel of poly-18 reactive, I-A^d restricted T cell hybridomas derived from BALB/c mice.

2. Assess results for dominant V gene usage that may be associated with poly-18 reactivity.

3. Determine the J region usage and junctional diversity associated with the two dominant V genes in the poly-18 specific hybridomas.

4. Attempt to draw conclusions regarding structure-function correlations between V gene usage patterns and functional response patterns known for the hybridomas.

Subsequent analysis of the BALB/c poly-18 response focuses on the J region usage and junctional diversity associated with the two dominant V genes (Chapter 3). Specific objectives of this study are:

1 Extend the analysis of J region usage and junctional diversity associated with the two dominant V genes by analysing poly-18 reactive bulk T cell lines from BALB/c mice.

2. Assess the role of the J regions and junctional diversity in conferring poly-18 + I-A^d reactivity.

Our second study deals with the use of a particular Vβ gene in the poly-18 T cell response in DBA/2 mice (Chapter 4). The

conceptual framework for this study came from two observations, one reported in the literature and one stemming from our analysis of the BALB/c poly-18 T cell receptor repertoire. Specifically, it was noted that in strains of mice like DBA/2 that express the minor lymphocyte stimulatory antigen-1^a (Mls-1^a), Vβ6 bearing thymocytes are clonally deleted during T cell maturation in the thymus (116, 117). As Vβ6 is one of the dominant V genes used in the poly-18 response in BALB/c (Mls-1^b), we were interested in whether any Vβ6 T cells were available in the DBA/2 mouse to participate in a poly-18 specific T cell response. On a more general level, we were interested in Vβ6 recognition of Mls-1^a and how it related to antigen recognition patterns seen in other antigen systems. Specific objectives of this study are:

1. Analyse Vβ6 hybridomas from BALB/c for Mls-1^a reactivity. Correlate reactivity with Vα gene usage and β chain junctional diversity.

2. Generate poly-18 specific T cell lines from DBA/2 mice. Determine if Vβ6 takes part in the response.

3. Determine the J region usage and junctional diversity associated with Vβ6 T cells participating in the response.

4. Assess the role of the J regions and junctional diversity in conferring poly-18 + I-A^d reactivity in DBA/2 mice.

5. Assess the role of the J regions and junctional diversity in conferring Mls-1^a nonreactivity and the escape from clonal deletion in DBA/2 mice.

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Chapter 2

T Cell Receptor Gene Usage in a panel of BALB/c poly-18 specific T cell Hybridomas[1]

T cells recognize processed antigen in association with major histocompatibility complex (MHC) molecules on the surface of antigen presenting cells (reviewed in 1 and 2). The antigen receptor on the surface of most T cells is a heterodimeric glycoprotein, in which the α and β chains are linked by disulphide bonds (1, 2). The antigen-MHC specificity of T cells is dictated by the $\alpha\beta$ T cell receptor (TCR). This was clearly demonstrated in transfection experiments, in which the genes encoding the α and β chains of the T cell receptor were shown to be both crucial and sufficient to transfer the antigen/MHC specificity of the donor T cell (3-5).

A number of antigen systems have been used to characterize the complex interactions between the trimolecular complex of the TCR, MHC and antigen. It seems that the antigens that show dominant V gene usage fall into two categories. The first group, characterized by immunodominant peptides of cytochrome c (6-8), myelin basic protein (9-11), and the hapten TNP on H-2K^b (12, 13)

1. A version of this chapter has been submitted for publication, Kilgannon P.,Z. Novak, M. Sadelain, J. Ratanavongsiri, T. Dillon, B. Singh, and A. Fotedar. 1990.

show limited heterogeneity with respect to the T cell repertoires that recognizes them. Reactivity to the other group of antigens, now referred to as "superantigens", is associated with the expression of particular Vβ genes. These include the Mls antigens (Mls-1a and 2a)(14-18), an unidentified self antigen presented on I-E molecules (19, 20) and Staphylococcal enterotoxins (21, 22). The junctional diversity in the β chain and the TCRα chain play a relatively smaller role in responses to these antigens. The question has arisen as to the nature of the TCR interaction with these two types of antigens. Recognition of peptide fragments is thought to involve numerous components of the TCR that make up the antigen binding cleft (23). Some speculate that the superantigens bind the TCR Vβ outside the cleft, thereby diminishing the role of junctional diversity and the α chain (24). Part of the difficulty with studying the superantigens is that the antigenic entities are not known, especially in the case of the Mls antigens.

In this communication, we report the characterization of the T cell repertoire to a synthetic peptide (poly-18) that has some properties of both antigen types. Poly-18 is an α-helical synthetic polypeptide antigen of defined sequence and conformation , and is a polymer of the monomeric 18 mer sequence EYK(EYA)5 (25). Immune response to this antigen is under Ir gene control (26). H-2^d strains are responders while H-2^b and H-2^k are nonresponder strains. We have characterized the TCR V gene usage in a panel of 16 T cell hybridomas from BALB/c, all specific for poly-18/I-A^d. Two dominant V genes emerged from this analysis, Vβ6 and Vα11. We find that Vβ6 is expressed by 50% of the poly-18 specific

hybridomas while Vα11 is expressed by 25%. The Vβ6 and Vα11 are expressed in mutually exclusive groups, thus accounting for 75% of the repertoire. There are a large number of other chains that can associate with each of the dominant V gene chains. The junctional diversity associated with the 2 dominant V genes in the panel of T cell hybridomas has also been ascertained. The junctional diversity and J region usage are heterogeneous in both dominant V genes used in the poly-18 T cell response.

Materials and Methods

Cells and Reagents

BALB/c mice were immunized with 30 μg of poly-18 emulsified with complete Freund's adjuvant. Popliteal lymph node T cells were used to generate poly-18 specific T cell lines by standard methods (26). The antigen reactivity of the T cell lines was tested every week. After 4-6 weeks of *in vitro* culture, the T cell lines were used to generate hybridomas or RNA was prepared for Northern analysis. T cell hybridomas were generated by fusing these T cell lines as described earlier (26) with BW5147 cells. T cell hybridomas were cloned twice by limiting dilution. The antigen/H-2 induced IL-2 release assay was used to determine the antigen specificity of the T cell hybrids. The reactivity patterns of the T cell hybridomas was determined using a panel of synthetic poly-18 variant peptides and a number of strains as antigen presenting cells (27). All the T cell hybridomas used in this analysis are restricted to $I\text{-}A^d$. This was shown by the ability of the anti-IA^d monoclonal antibody MKD6 to block antigen/MHC induced cytokine release. In addition, B10.GD spleen cells which only bear $I\text{-}A^d$ but not $I\text{-}E^d$ can act as effective antigen presenting cells for all these hybridomas (data not shown). The poly-18 specific T cell hybridomas referred to in this study were generated in Dr. B. Singh's laboratory by Z. Novak.

The T cell receptor V gene specific probes were cDNA or genomic clones cloned into pUC18/19 either in our laboratory or obtained from Dr. L. Hood's laboratory at Caltech (Pasadena, Ca). Alternatively, 30 mer synthetic oligonucleotides were synthesized at

the Regional DNA Synthesis Laboratory at the University of Calgary. The TCR V gene probes used in this study are listed in Table 2.1.

cDNA Cloning and Sequencing α and β Chains of the TCR

Total cellular RNA was isolated from 10^9 cells by the conventional guanidium-isothiocyanate and cesium chloride method (28). Poly A^+ RNA was recovered by oligo-dT cellulose affinity chromatography. The first strand synthesis was generated using an oligo-dT primer and reverse transcriptase and the second strand using DNA polymerase I and RNase H. The methylated blunt ended double-stranded (ds) cDNA was ligated to EcoRI linkers. Subsequent to EcoRI digestion the dscDNA was size selected on agarose gels , purified by spermine precipitation, and cloned into λgt10 (Stratagene, La Jolla, CA). Phage particles were packaged using GIGAPACK in vitro packaging extracts (Stratagene, La Jolla, CA). Approximately 200,000 plaques were screened by *in situ* hybridization using Cα and Cβ probes. The Cβ probe has b described earlier (29) and Cα probe was obtained by screen cDNA library (made from a beef insulin specific T cell hybrid with a Cα specific synthetic oligonucleotide (Martien van H unpublished results). Insert DNA from the positive clones was ligated into M13mp18 and M13mp19 for standard dideoxy sequencing (30).

Northern and Southern Analysis

Total cellular RNA was isolated from ~10^8 cells by a rapid method (31). 10-20 μg of RNA from each sample was run on 1%

agarose gels. After transferring to nitrocellulose, the RNA was hybridized as outlined below. Nitrocellulose membranes were prewashed in 3xSSC at 65°C for 1 hour. Prehybridization was for 3-4 hours at 65°C in 3xSSC/10x Denhardt's. Hybridization was conducted overnight at 65°C in 10x Denhardt's, 1M NaCl, 50mM Tris(pH8), 10mM EDTA, 0.1% SDS, 100mg/ml denatured salmon sperm DNA and oligolabelled probes (H). Filters were washed in 2xSSC at room temperature for 10-20 min., 2xSSC/1% SDS at 65°C for 1 hour and a final wash in 2xSSC at room temperature for 10-20 min., and exposed to X-ray film for 1-3 days.

High molecular weight genomic DNA was isolated from T cell hybridomas (28) and after digesting 20-25 μg of genomic DNA with restriction enzymes was resolved on 1% agarose gels. DNA was transfered to nylon membrane (Gene Screen Plus, NEN) by the alkaline transfer method (32). The membranes were washed in 3X SSC at 65°C for 1 hour, then prehybridized in 10% dextran sulfate, 1% SDS, 1 M NaCl at 65°C for 3-4 hours. Hybridization was done in the same solution as the prehybridization except 100mg/ml denatured salmon sperm DNA and oligolabelled probe were included. The membranes were incubated 14-16 hours in a circulating waterbath at 65°C. Filters were washed in a similar manner as the Northerns described above.

Oligonucleotide probes were labelled by 5' end kinasing (28). The conditions for hybridization with oligonucleotide probes and subsequent washing in the presence of tetramethylammonium chloride are described elsewhere (33).

FACS Analysis

Cell surface expression of Vβ6 and Vβ8 gene products was assessed by indirect immunofluorescence staining followed by FACS analysis. Hybridomas 44-22-1 specific for Vβ6 (15) and F23.1 specific for Vβ8.1, Vβ8.2 and Vβ8.3 (34) were kindly provided by Drs. H. Hengartner and M. Bevan respectively. Aliquots of 10^6 T cells in exponential growth were stained using culture supernatants at a 1:2 dilution followed by the fluoresceinated goat anti-rat IgG (for 44-22-1) and antimouse IgG (for F23.1) as the second reagent (Tago Inc., Burlingame, Ca.). Flow cytometric analysis of 10^4 cells was carried out using a Coulter Epics V cell sorter. The author would like to acknowledge Michel Sadelain for doing the FACS analysis.

Junctional Diversity Analysis

RNA from individual T cell hybridomas was isolated by standard guanidium isothiocyanate and cesium chloride methods (28). Conditions for cDNA and PCR were essentially as described by Erlich (35). First strand cDNA synthesis was carried out using 1-2μg total cellular RNA, 100 pmoles random hexamers (primer), 0.25 mM dNTP's, 0.1 volume 10X PCR buffer (KCl; 0.5M, Tris-8.3; 0.2M, MgCl; 25mM, BSA; 1mg/ml) in a 20μl volume. The RNA solution was incubated at 95°C for 1 minute then quick cooled on ice before 200 units of MuLV Reverse transcriptase (Gibco BRL) was added. The reaction was incubated at room temperature for 10 minutes followed by 37°C for an additional 60 minutes. The entire RTase reaction was

used in the subsequent PCR step. Additional 10X PCR buffer(8μl) and dNTP's (1 mM, final conc.) were added to the RTase reaction as well as 0.25μM of each of the two primers (Vβ6 and Cβ or Vα11 and Cα-sequences shown in Figure 2.1). The final reaction volume was 100 μl, to which 4 units of Taq DNA polymerase (Promega) was added. The reaction was overlayed with parafin oil to prevent evaporation. Temperature cycling steps included a denaturation step (92°C; 2 min.), an annealing step (55°C; 1 min.) and and extension step (72°C; 1 min.). This cycle was repeated 35 times. A portion of the PCR reaction was run on a 2% agarose gel and the DNA band was purified.

The purified PCR product was phosphorylated with polynucleotide kinase (PNK) and blunt-ended with T4 DNA polymerase before being ligated into the SmaI site of pUC 18. Reaction conditions were essentially as described in Maniatis et al. (28). Ligation reactions were transformed into competent TB-1 cells and plated on LB agar plates containing ampicillin and X-gal. The bacteria were incubated at 37°C overnight. Recombinant (white) colonies were picked and plasmid DNA mini-preparation's carried out. The Sequenase DNA sequencing kit (United States Biochemicals) was use for double stranded sequencing as per the manufacturer's instructions. A nested Cα or Cβ primer was used in place of the universal primer in the sequencing reactions (shown in Figure 2.1).

Results

We have assessed the TCR variable gene usage in a panel of T cell hybridomas specific for poly-18 generated from BALB/c mice. The panel was composed of hybridomas from fusions of 6 separate bulk T cell lines. Poly-18 specific hybridomas were rigorously characterized for antigen fine specificity, H-2 restriction and alloreactivity by using a panel of synthetic poly-18 variant peptides and spleen cells from various mouse strains as antigen presenting cells (27).

The α and β chains of the T cell receptor (TCR) from 3 poly-18 specific T cell hybridomas were analyzed by cDNA cloning and dideoxy sequencing (Fig. 2.2a and b). The nomenclature for Vα (36,37), Jα(37,38), Vβ(39-41), Dβ(42) and Jβ(43) is as described. The T cell hybridoma B14 uses Vα1, JαTA65, Vβ6, Jβ2.7 gene segments. The Vα1 gene family has 5 members (36-38. 44). The B14 Vα gene segment is identical to TA84 (36). The T cell hybridoma B15 uses Vα11, JαTT11, Vβ2, and Jβ1.3 gene segments. Both the α and β sequences are truncated at the 5' end due to EcoR1 sites in the V regions. The Vα11 gene family has at least 2 described members (6). The Vα11 gene used by B15 is a new family member. The third hybridoma, B16 uses Vα3, JαTA27, Vβ1 and Jβ1.6. The Vα expressed in B16 belongs to a 5 member family (36, 44-46) and is identical to AF3 (44). Comparison of amino acid sequences in different regions of the α and β chains did not reveal any conserved sequences that might account for poly-18 reactivity in these hybridomas.

Since the V and J elements sequenced in these initial T cells were all different, we decided to extend the study by doing a Northern analysis on a larger panel of poly-18/I-A^d reactive T cell hybridomas using 10 Vα and 11 Vβ specific probes (listed in Table 2.1). In addition, we did a flow cytometric analysis with 2 V region specific monoclonal antibodies, 44-22-1 (Vβ6 specific) and F23.1 (Vβ8.1, 8.2, 8.3 specific) on our panel of T cell hybridomas. The Northern data was completely concordant with the FACS data (Table 2.2). Results of Vα11 and Vβ6 hybridizations with RNA from the hybridomas are shown in Figure 2.3a and b. In addition, we did a Southern analysis of rearrangement patterns with some of the T cell hybrids using Vα1 and Vβ1 probes since they are also expressed by the fusion partner BW5147. T cell hybridomas B1 and B7 are designated as $V\beta1^+$ based on this analysis. They showed the same rearrangement pattern with Vβ1 as each other in addition to the BW5147 pattern (Figure 2.4). The summary table of V gene usage by the poly-18 specific T cell hybridomas was based on composite data generated from our cDNA cloning/sequencing, Northern, Southern and FACS analysis (Table 2.2). T cells participating in the poly-18 response can be divided into 3 categories based on their V gene usage. First approximately 50% of poly-18 reactive T cells express Vβ6. In our panel, the following 8/16 T cell hybridomas constitute this group; B2, B3, B4, B5, B8, B11, B14 and B17. Second, Vα11 bearing T cells represent approximately 25% of the repertoire. The following 4/16 T cell hybridomas express Vα11; B1, B6, B9 and B15. This group does not overlap with Vβ6 as no examples of Vβ6/Vα11 hybrids were found. Thus a full 75% of the T cell

repertoire is accounted for by receptors utilizing either Vβ6 or Vα11. Interestingly, a broad range of Vα genes can combine with Vβ6. We noted the use of Vα1, 5, 7, 8, and an unknown Vα in the T cells that used Vβ6. Similarly, a broad range of Vβ genes can combine with Vα11 to generate poly-18 reactivity. These include Vβ1, 2, and 8. The third group is a mixed group that uses neither Vβ6 nor Vα11 in their receptors. Despite the prevalent use of Vβ6 and Vα11, the heterogeneity of the overall poly-18 response is notable. In 16 T cell hybridomas, 12 different α/β combinations are seen.

The dominant V genes expressed in our panel were also detectable in RNA from the bulk T cell lines used to generate the hybridomas (data not shown). V genes not found in our panel of hybridomas were not detected in the Northern analysis of the bulk T cell lines. In addition, approximately 40% of CD4 expressing blast cells in 3 poly-18 specific T cell lines tested were positive for Vβ6 (data not shown). This suggested that the pattern of V gene expression seen in the poly-18 specific T cell hybridomas accurately reflected the situation in the bulk T cell lines.

We analysed the junctional diversity associated with each of the dominant V genes, Vβ6 and Vα11. Specific primers in the V and C regions were used to amplify and clone Vβ6 and Vα11 sequences from the RNA from appropriate T cell hybridomas (oligonucleotides used are shown in figure 2.1). Generation of sequence data from these hybridomas confirms the dominant V gene usage. Junctional diversity from T cell hybridoma B15 as determined from cDNA cloning and sequencing analysis is listed with the other Vα11 hybridomas for comparison. In 4 Vα11 hybridomas, 3

different Jα regions are seen (Fig. 2.5). At least two different members of the Vα11 family are being used in the poly-18 response. A notable difference between these members is the presence of an extra CGG-Arg codon in the Vα11 used by T cell hybrids B6 and B15. the significance of this codon is not readily apparent at this time. The junctional diversity associated with Vβ6 in the panel of hybridomas is also diverse. In 8 Vβ6 hybridomas, we see 6 different rearrangement patterns using 5 different Jβ's (Fig. 2.6). T cell hybridomas B2, B4 and B5 all use Jβ2.4 with the same junctional sequences. B3 and B17 both use Jβ1.3 but differ in the junction region. Hybridomas B8, B11 and B14 use Jβ2.3, 1.2 and 2.7 respectively.

Discussion

What conditions lead to apparent dominant V gene usage in antigen specific responses? The answer to this question depends on the nature of the TCR interaction with the antigen/MHC complex. Two likely scenarios exist. First, if a particular V region alone confers general reactivity to the antigen while the junctional diversity, J region and other chain of the TCR play a secondary role, then the precursor frequency of reactive T cells bearing that V gene will be high. As the importance of the junctional diversity, J regions and other chain diminishes, the precursor frequency of responsive T cells will increase accordingly. This situation has been described for a number of antigens, now referred to as superantigens (14-22). A functional definition of a superantigen has emerged based on this type of recognition pattern. If on the other hand, a precise requirement for specific motifs in one or both TCR chains exists then the antigen specific T cell repertoire will be relatively narrow. In this case the same few clones will dominate the cultures. This situation has been described for a number of nominal antigens (6-13).

The dominant V gene usage observed in the poly-18 repertoire clearly is not the result of particular clones dominating the immune response. Diversity in the TCR's that recognize poly-18/I-A^d exists at two levels. First, there is a large number of other chains capable of pairing with the dominant V gene chains. In 4 Vα11 hybridomas, 3 different β chains are seen. In 8 Vβ6 hybridomas, 5 different α chains are seen. Secondly, the junctional diversity and J region usage

associated with the dominant V genes is very large. Analysis of a limited number of hybridomas makes it difficult to estimate the extent of junctional diversity and J region usage associated with Vβ6 and Vα11. However, the trend in the poly-18 system is toward conserved use of certain V genes. At the same time, there doesn't appear to be the same selective pressure on the junctional diversity and J region usage associated with the dominant V genes or on the other TCR chains that pair with the dominant V gene chain. Similar to an evolutionary argument, we suggest that this lack of selective pressure implies a diminished role in conferring overall reactivity to poly-18. It goes without saying however, that the J regions and the other TCR chain that associates with the dominant V gene chains affect the fine specificity of the response. Functional analysis of cytochrome c specific T cell clones revealed the importance of junction sequences in determining unique fine specificity profiles (47). Likewise, when the antigen fine specificity analysis of our panel of poly-18/I-A^d reactive T cell hybridomas was carried out, a great deal of microheterogeneity was observed (27). Thus the findings of the functional analysis can be explained by the molecular analysis of the TCR's involved.

The observations in the poly-18 system differ from other nominal antigen systems described. Peptide antigen systems best characterized for TCR gene usage are cytochrome c and myelin basic protein. In B10.A mice (I-E^k), the T cell response to the C-terminal region (81-104) of pigeon cytochrome c is dominated by Vα11.1/Vβ3 T cell receptors (6-8). In B10.S(9R) mice (I-E^s) the response is dominated by Vα10/Vβ1 TCR's (48). In each case a limited number

of D-Jβ and Jα regions are used. T cells respond to the N-terminal peptide (1-9) of myelin basic protein (MBP) in association with I-A^u. Two dominant Vβ genes (Vβ8.2 & 13) appear to assort with one of two dominant Vα genes (Vα2.3 & 4)(9-11). There is only limited junctional diversity and J region usage in the response. Enough of the response is dominated by Vβ8.2 bearing receptors that treatment with MAb F23.1 (specific for Vβ8) drastically reduces the incidence of EAE in these mice. In the TNP/H-$2K^b$ response, 40% of the independently isolated T cells use identical TCRα and β chains (12,13). The T cell response to the λ repressor c1 protein shows predominant use of Vα2 and to a lesser degree, Vβ1 (49,50). Limited junctional diversity is detected in the Vα2 chains. Vβ1 is rearranged to Jβ2.1 in most cases with an apparent conservation of a glutamic acid residue in the junction. These examples illustrate the importance of the whole antigen binding cleft in some antigen responses. While critical amino acids can be identified in the junction regions of some of these TCR's (51,52), clearly elements on both chains are being selected for simultaneously. This is in contrast to poly-18 where the dominant V genes alone may be largely responsible for conferring antigen specificity. The T cell response to other antigens shows a variety of patterns. Some appear quite heterogeneous in the number of different TCR α/β combinations associated with a given response. The 25% of the poly-18 response that does not express either Vβ6 or Vα11 may parallel some of these complicated response patterns. The responses to insulin (44,53), lysozyme (54), the hapten AED (55) and the allo antigen I-A^{bm12} (56) fall into this category. No simple generalizations about what type of

antigen will elicit what type of T cell repertoire can be made at this time.

The manner in which TCR's interact with the antigen/MHC complex appears to be different for different antigens. Clearly the relative role of each component of the TCR will vary from case to case. At one end of the continuum are the superantigens. They elicit a response from almost all T cell bearing a particular Vβ (14-22). Pullen et al. have identified residues on Vβ8.1 that are critical for Mls-1^a recognition (57). A closer look at the Mls-1^a/Vβ6 system however has revealed that some immunologically competent $Vb6^+$ T cells do escape deletion in the DBA/2(Mls-1^a) thymus (Chapter 4). This implies that while the Vβ6 gene is the predominant determinant of Mls-1^a reactivity, the D-Jβ region and TCRα chain still play a minor role. Others have noted that not all Vβ6 T cells are Mls-1^a reactive (15). At the other end of the continuum are antigens like the C-terminal peptide of cytochrome c, the N-terminal peptide of myelin basic protein or the hapten TNP/H-$2K^b$. These are examples where there are strict requirements for multiple elements in the TCR before antigen reactivity is seen.

The poly-18 response appears to be a mixture of reactivity patterns. The two dominant V genes account for 75% of the BALB/c repertoire. Interestingly, one is a Vα (α11) and the other is a Vβ (β6). Within each of these groups the V genes are associated with an enormous amount of diversity at the junctions and in the J region usage. In addition, there appears to be a large number of other chains that can associate with the dominant V gene chains to yield poly-18/I-A^d reactivity. This could imply that the dominant V genes

play a large role in determining reactivity to poly-18. Thus in the continuum of reactivity patterns discussed above, this portion of the poly-18 response is closer to the pattern for superantigens. This is not to say that poly-18 is a superantigen, only that the relative involvement of different components of the TCR in the response parallels some features of the superantigen systems. The simple repeating nature of poly-18 may influence the type of T cell response generated. The monomer does not require processing, ruling out the possibility of differential processing (58). It is possible that the antigen binds the MHC in a number of ways. This has been shown to be the case in the poly-18 system (59). It has also been suggested that there is a degree of flexibility in how cytochrome c peptides are presented by the restricting element (47). The T cell response to sperm whale myoglobin and the hapten pABA share some of the features of the poly-18 system. In the myoglobin response, one dominant Vβ gene (Vβ8.2) can pair with a number of different TCRα chains (60,61). This might argue for the primary role of the β chain in conferring reactivity to myoglobin. The myoglobin system differs from the poly-18 system however in the junctional diversity and J region usage associated with the dominant Vβ gene. Only one Jβ region is seen and the junctional diversity is very limited. The T cell response to the hapten pABA is dominated by Vα3 (45). A number of β chains can be associated with Vα3 to generate pABA specificity. The Vα3 gene is suggested to confer reactivity to pABA on at least 3 different MHC molecules. Thus, dominant V gene usage associated with a primary role in conferring antigen reactivity, can be either Vα or Vβ. The poly-18 T cell

repertoire is dominated by an example of each. The remaining 25% of the poly-18 repertoire is likely composed of a mixture of TCR's. These may represent reactivity patterns closer to those seen in other nominal antigen systems.

Our current conception of how TCR interacts with antigen-MHC complexes comes from models of TCR and MHC structure. Crystallographic data is available for class I MHC (62,63). Class II is generally thought to share many of the structural features (64). This premise is supported by the observation that TCR recognition of Class I restricted antigens is not fundamentally different from class II restricted antigens. The model of TCR structure is based on the structure of immunoglobulin molecules (65,66). The hypervariable regions in the TCR are not as discrete as those identified in immunoglobulins therefore predicting contact sites along the TCR is more difficult. Superantigens can activate T cells via the Vβ portion of the TCR while most nominal antigens require numerous components on the TCR for reactivity. This implies that the TCR can interact with antigen in a number of ways, all of which lead to activation and clonal expansion. The apparent variety of TCR reactivity patterns seen for the different antigen systems may be an indication of the complexity and flexibility in T cell recognition.

Our ability to predict what type of T cell repertoire an antigen will elicit is still very poor. A clear understanding of this will greatly help fields like synthetic vaccine design and intervention in autoimmune diseases. The T cell repertoire in numerous antigen systems must be analyzed so that we may get a more accurate impression of the truly complex nature of T cell responses. Until

adequate structural data becomes available we have to continue to make inferences about the interactions of TCR with antigen and MHC using model antigen systems.

Table 2.1 Panel of T cell receptor Variable (V) region DNA probes.

Gene	Source	Length(bp's)
Vα1	cDNA	400
Vα2	cDNA	200
Vα3	oligo	30
Vα4	cDNA	275
Vα5	cDNA	241
Vα6	cDNA	400
Vα7	cDNA	260
Vα8	cDNA	300
Vα9	cDNA	130
Vα11	genomic	2400
Vβ1	cDNA	275
Vβ2	genomic	530
Vβ3	cDNA	260
Vβ4	cDNA	300
Vβ5	cDNA	230
Vβ6	cDNA	400
Vβ7	cDNA	300
Vβ8	cDNA	225
Vβ10	cDNA	375
Vβ14	cDNA	250
Vβ16	cDNA	300

Table 2.2 T Cell Receptor Variable Gene usage in BALB/c anti Poly-18 T cell Hybridomas

ORIGIN	HYBRIDOMA	Vα	METHOD[1]	Vβ	METHOD[1]
BALB/c	B2	5	a	6	a,b
	B3	8	a	6	a,b
	B4	--	a,d	6	a,b
	B5	5	a	6	a,b
	B8	--	a	6	a,b
	B11	7	a	6	a,b
	B14	1	a,c	6	a,b,c
	B17	ND	ND	6	b
	B1	11	a	1'	a,d
	B6	11	a	8	a,b
	B9	11	a	8	a,b
	B15	11	a,c	2	a,c
	B7	--	a,d	1'	a,d
	B12	6	a	2	a
	B13	--	a,d	--	a,d
	B16	3.2	c	1	a,c

[1] Methods used to determine V gene usage: a. Northern analysis with V region specific probes, b. FACS analysis with 44-22-1 (Vβ6 specific) or F23.1 (Vβ8.1, 8.2 and 8.3 specific), c. cDNA cloning and sequencing, d. southern analysis with Vα1 or Vβ1 probes. Those T cell hybridomas not hybridizing with any of the available probes are indicated with "--". ND - not done.

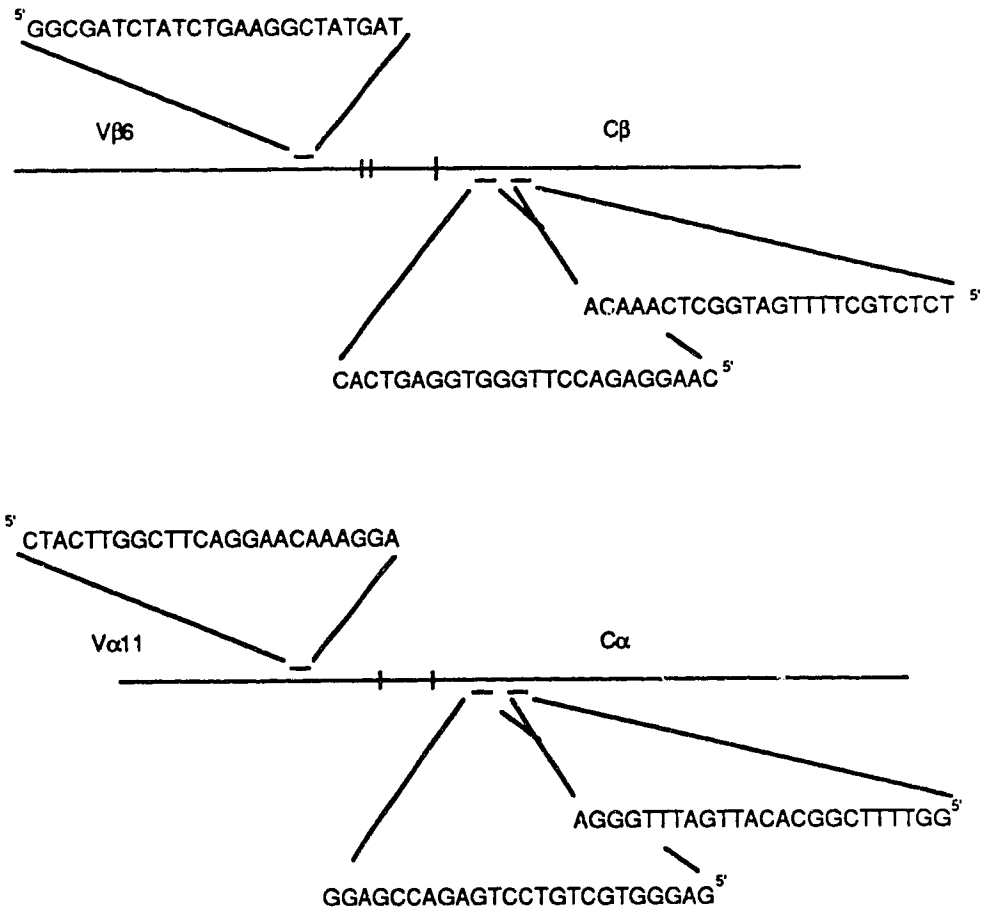
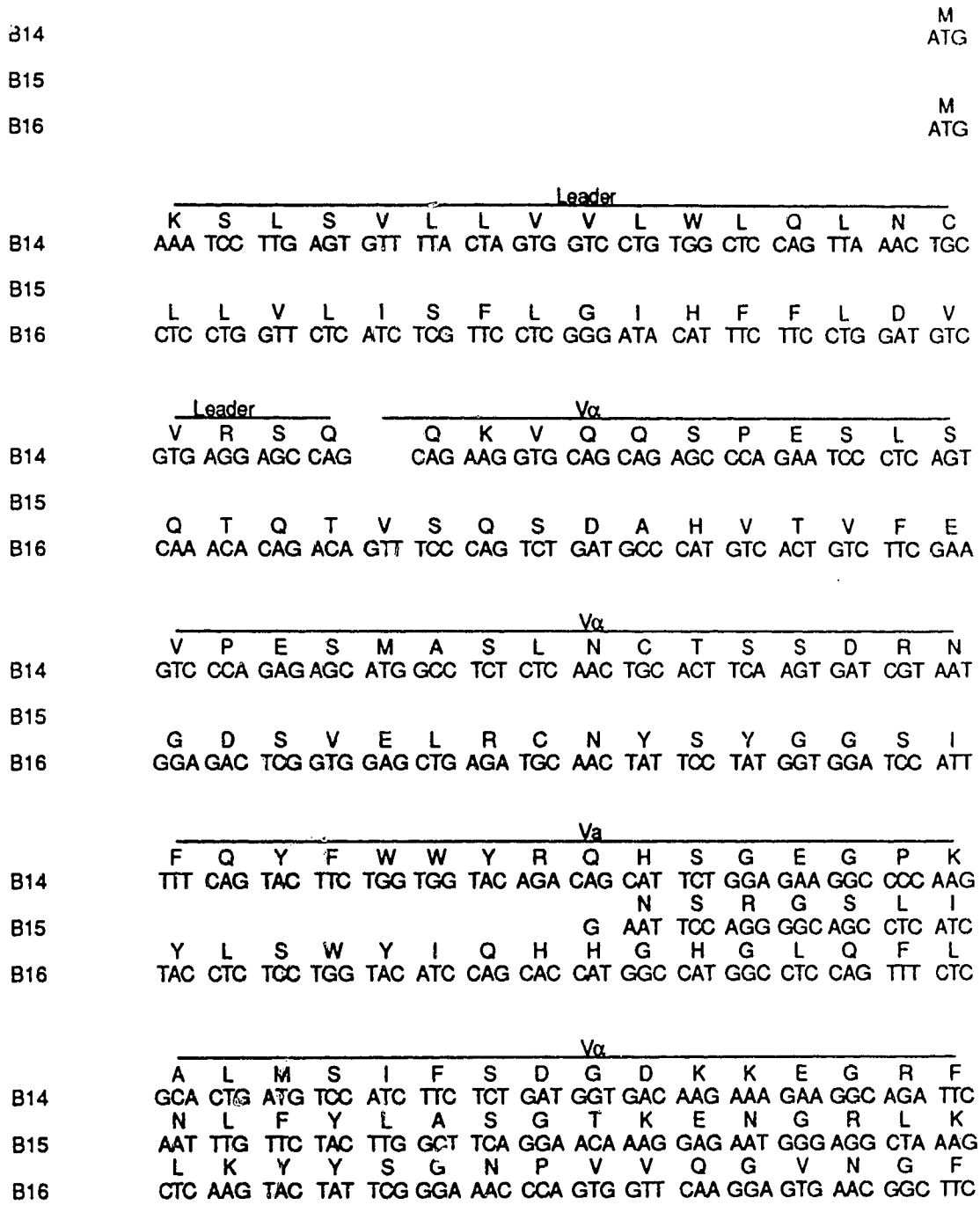


Figure 2.1. Oligonucleotides used in Polymerase Chain Reaction to amplify Vα11 and Vβ6 junction sequences.

Variable region oligonucleotides correspond to the sense strand while C region oligonucleotides are antisense. The 3' C region oligonucleotides were used in the primary amplification and the 5' C region oligonucleotides were used in the sequencing reactions.


B14
M
ATG
B15
B16
M
ATG
Leader
K S L S V L L V V L W L Q L N C
B14 AAA TCC TTG AGT GTT TTA CTA GTG GTC CTG TGG CTC CAG TTA AAC TGC
B15
L L V L I S F L G I H F F L D V
B16 CTC CTG GTT CTC ATC TCG TTC CTC GGG ATA CAT TTC TTC CTG GAT GTC
Leader
Vα
V R S Q Q K V Q Q S P E S L S
B14 GTG AGG AGC CAG CAG AAG GTG CAG CAG AGC CCA GAA TCC CTC AGT
B15
Q T Q T V S Q S D A H V T V F E
B16 CAA ACA CAG ACA GTT TCC CAG TCT GAT GCC CAT GTC ACT GTC TTC GAA
Vα
V P E S M A S L N C T S S D R N
B14 GTC CCA GAG AGC ATG GCC TCT CTC AAC TGC ACT TCA AGT GAT CGT AAT
B15
G D S V E L R C N Y S Y G G S I
B16 GGA GAC TCG GTG GAG CTG AGA TGC AAC TAT TCC TAT GGT GGA TCC ATT
Va
F Q Y F W W Y R Q H S G E G P K
B14 TTT CAG TAC TTC TGG TGG TAC AGA CAG CAT TCT GGA GAA GGC CCC AAG
N S R G S L I
B15 G AAT TCC AGG GGC AGC CTC ATC
Y L S W Y I Q H H G H G L Q F L
B16 TAC CTC TCC TGG TAC ATC CAG CAC CAT GGC CAT GGC CTC CAG TTT CTC
Vα
A L M S I F S D G D K K E G R F
B14 GCA CTG ATG TCC ATC TTC TCT GAT GGT GAC AAG AAA GAA GGC AGA TTC
N L F Y L A S G T K E N G R L K
B15 AAT TTG TTC TAC TTG GCT TCA GGA ACA AAG GAG AAT GGG AGG CTA AAG
L K Y Y S G N P V V Q G V N G F
B16 CTC AAG TAC TAT TCG GGA AAC CCA GTG GTT CAA GGA GTG AAC GGC TTC

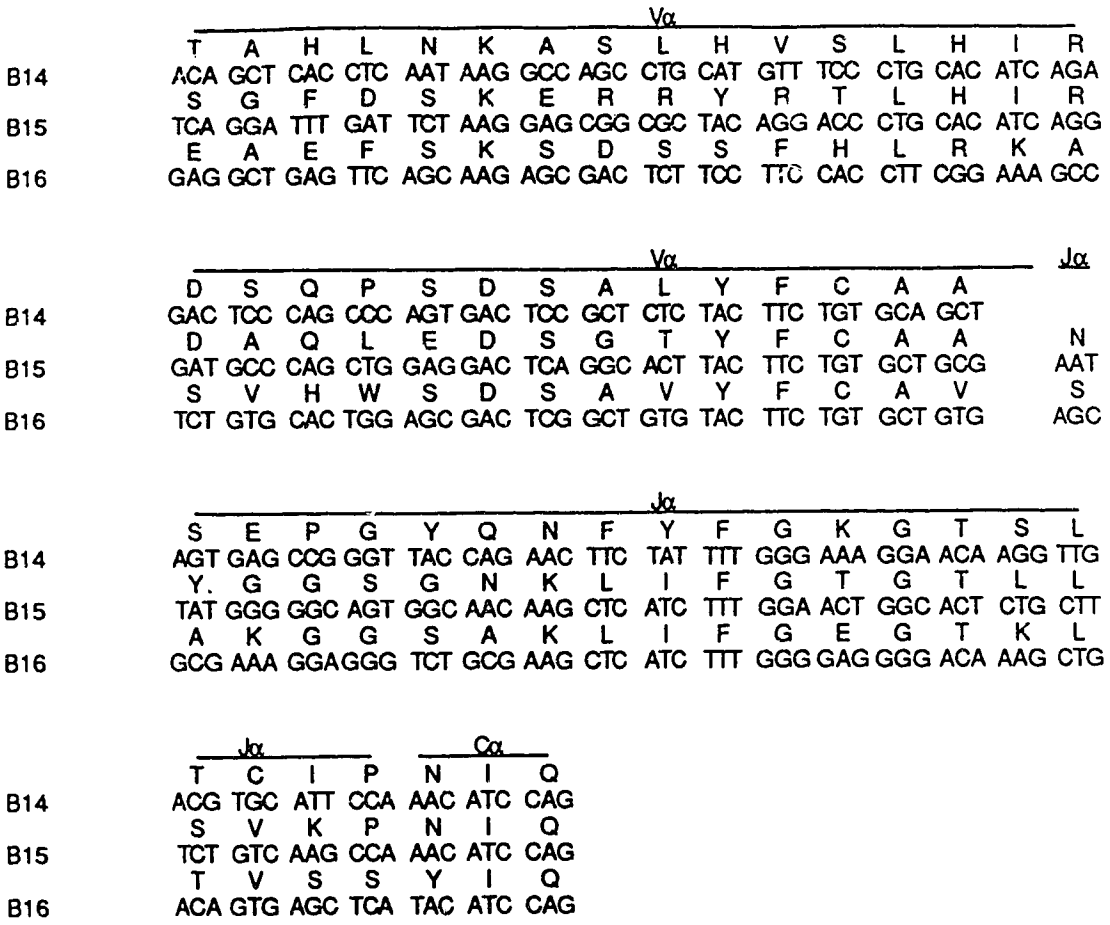
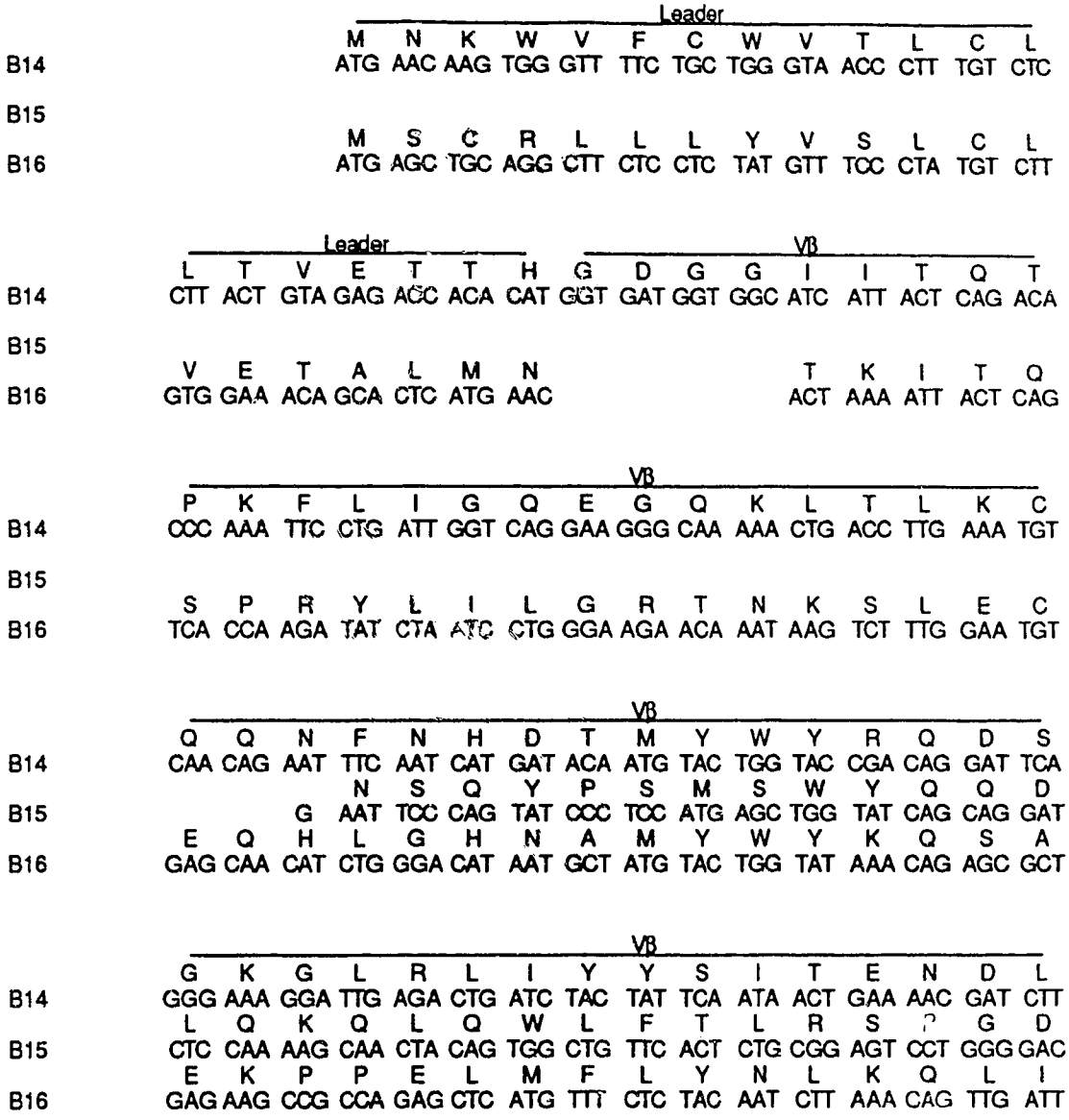


Figure 2.2a. cDNA sequence of TCRα chains from 3 poly-18 specific T cell hybridomas.

Single letter amino acid code is indicated above each codon. Sequences for T cell hybridomas B14 and B16 are shown from the translation start site. Sequence of the TCRα chain for T cell hybridoma B15 is truncated due to an Eco RI site in the V region. B14 uses Vα1 and JαTA65 (36). B15 uses a member of the Vα11 family not previously described and JαTT11 (38). B16 uses a member of the Vα3 family and JαTA27 (36,44).


Leader
M N K W V F C W V T L C L
B14 ATG AAC AAG TGG GTT TTC TGC TGG GTA ACC CTT TGT CTC
B15
M S C R L L L Y V S L C L
B16 ATG AGC TGC AGG CTT CTC CTC TAT GTT TCC CTA TGT CTT
Leader
Vβ
L T V E T T H G D G G I I T Q T
B14 CTT ACT GTA GAG ACC ACA CAT GGT GAT GGT GGC ATC ATT ACT CAG ACA
B15
V E T A L M N T K I T Q
B16 GTG GAA ACA GCA CTC ATG AAC ACT AAA ATT ACT CAG
Vβ
P K F L I G Q E G Q K L T L K C
B14 CCC AAA TTC CTG ATT GGT CAG GAA GGG CAA AAA CTG ACC TTG AAA TGT
B15
S P R Y L I L G R T N K S L E C
B16 TCA CCA AGA TAT CTA ATC CTG GGA AGA ACA AAT AAG TCT TTG GAA TGT
Vβ
Q Q N F N H D T M Y W Y R Q D S
B14 CAA CAG AAT TTC AAT CAT GAT ACA ATG TAC TGG TAC CGA CAG GAT TCA
N S Q Y P S M S W Y Q Q D
B15 G AAT TCC CAG TAT CCC TCC ATG AGC TGG TAT CAG CAG GAT
E Q H L G H N A M Y W Y K Q S A
B16 GAG CAA CAT CTG GGA CAT AAT GCT ATG TAC TGG TAT AAA CAG AGC GCT
Vβ
G K G L R L I Y Y S I T E N D L
B14 GGG AAA GGA TTG AGA CTG ATC TAC TAT TCA ATA ACT GAA AAC GAT CTT
L Q K Q L Q W L F T L R S P G D
B15 CTC CAA AAG CAA CTA CAG TGG CTG TTC ACT CTG CGG AGT CCT GGG GAC
E K P P E L M F L Y N L K Q L I
B16 GAG AAG CCG CCA GAG CTC ATG TTT CTC TAC AAT CTT AAA CAG TTG ATT

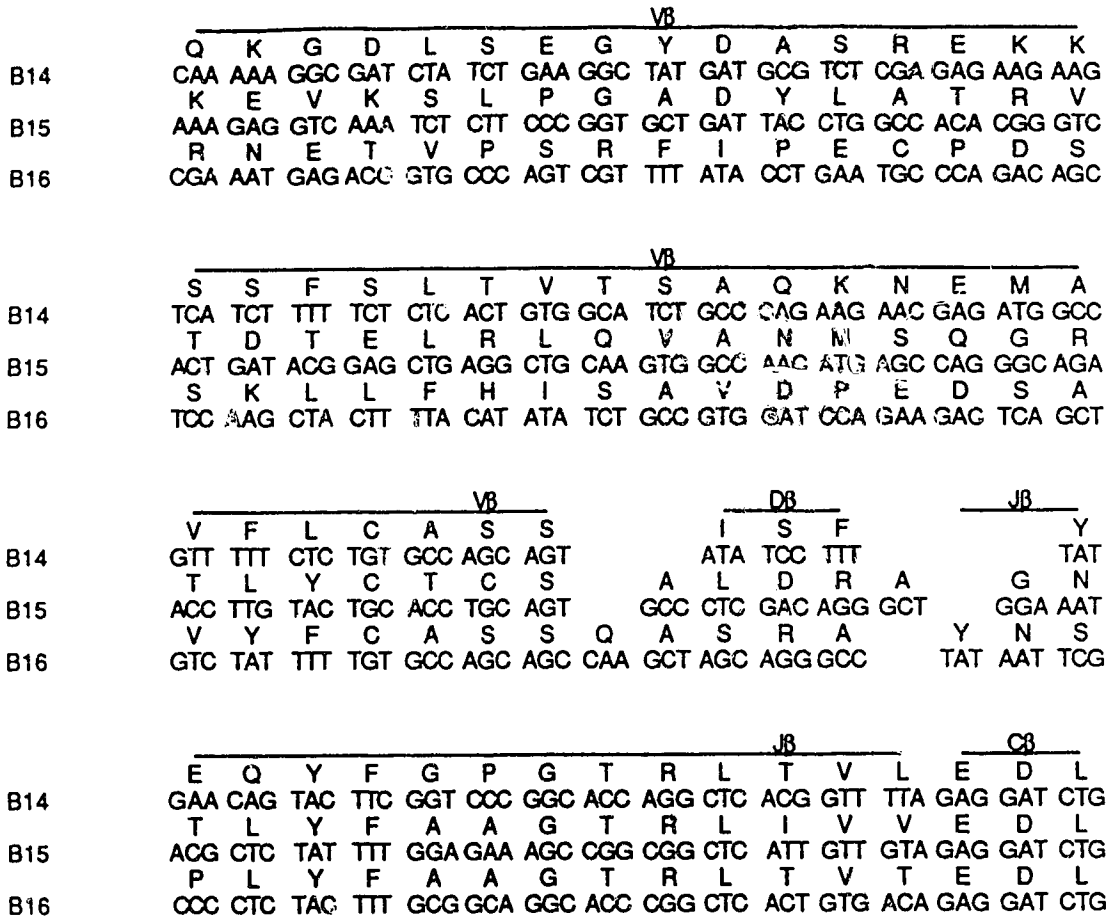


Figure 2.2b. cDNA sequence of TCRβ chains from 3 poly-18 specific T cell hybridomas.

Single letter amino acid code is indicated above each codon. Sequences for T cell hybridomas B14 and B16 are shown from the translation start site. Sequence of the TCRβ chain for T cell hybridoma B15 is truncated due to an Eco RI site in the V region. B14 uses Vβ6 and Jβ2.7. B15 uses Vβ2 and Jβ1.3. B16 uses Vβ1 and Jβ1.6.

a. Lane 1 2 3 4 5

b. Lane 1 2 3 4 5 6 7 8

Figure 2.3a and b. Northern analysis of poly-18 specific BALB/c T cell hybridomas with Vα11 and Vβ6 probes.

a. Vα11 analysis: Lane 1=B14 (negative control), 2=B15, 3=B9, 4=B6 and 5=B1.

b. Vβ6 analysis: Lane 1=B2, 2=B3, 3=B4, 4=B5, 5=B8, 6=B11, 7=B14 AND 8=B15 (negative control).

Lane 1 2 3 4 5

Figure 2.4. Southern analysis of Vβ1 rearrangement pattern of poly-18 specific BALB/c T cell hybridomas.

Genomic DNA was digested with Eco R1 and run on a 1% agarose gel. DNA was transferred to nylon membrane and hybridized with a labelled Vβ1 probe. Lane 1=B1, 2=B7, 3=B16 (expresses Vβ1, Jβ1.6), 4=BW5147 (fusion partner, expresses Vβ1) and 5=murine liver DNA (unrearranged).

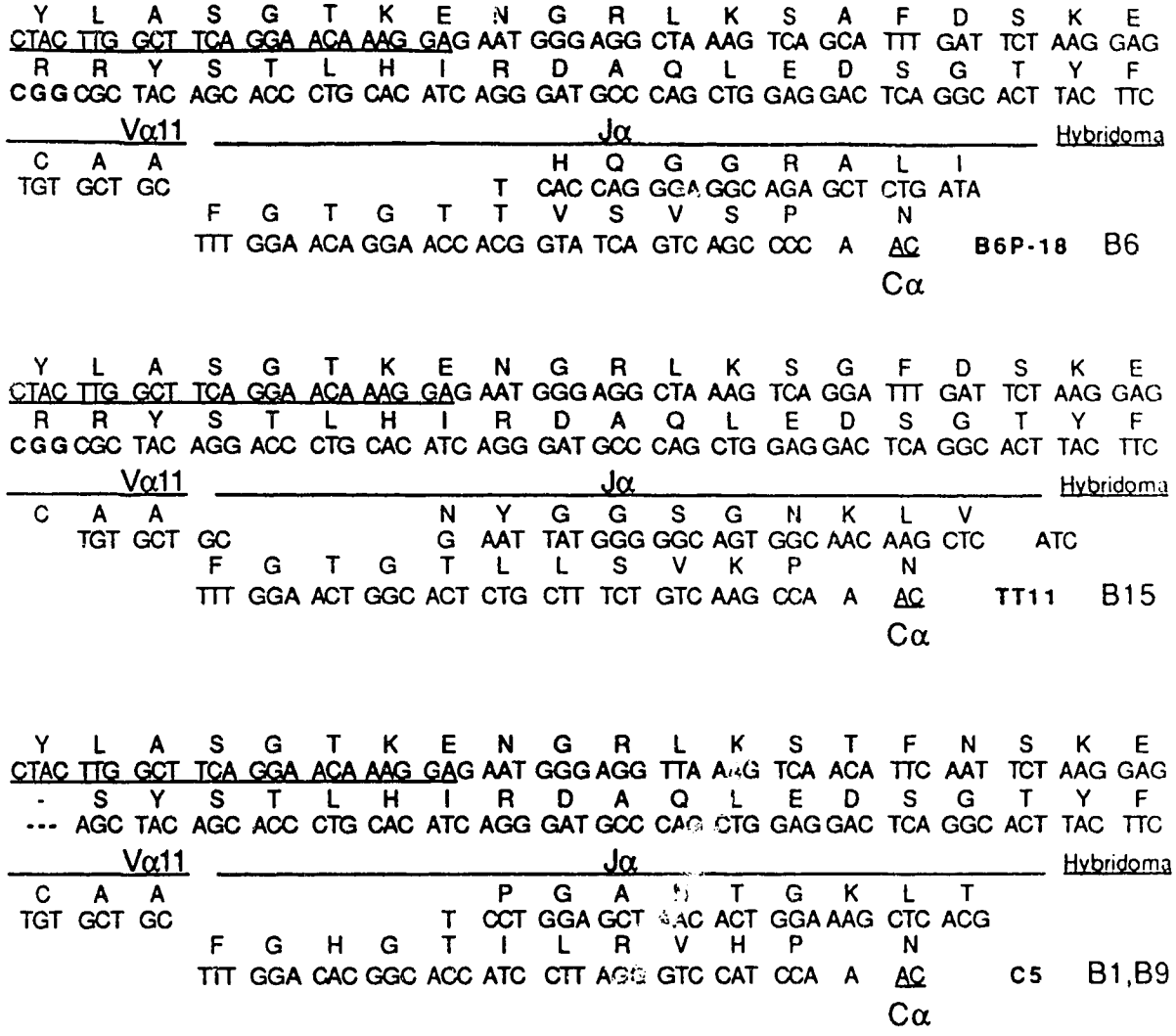


Figure 2.5. Junctional diversity and Jα region usage in Vα11$^+$ poly-18 reactive T cell hybridomas

Jα regions C5 and TT11 have been described previously (37,38). The region of Vα11 that is underlined in each sequence is one of the oligonucleotides used in the PCR to amplify this region. The extra codon (GCC) found in some Vα11 family members is highlighted in the figure.

Vβ6	Dβ	Jβ	T cell Name
C A S S TGT GCC AGC AG	T I G Q ATA GGA CAA	N S D Y T AAC TCC GAC TAC ACC-1.2	B11
C A S S TGT GCC AGC AG	C Q G Q L CAG GGA CAG CTT	S G N T L Y AGT GGA AAT ACG CTC TAT-1.3	B3
C A S S TGT GCC AGC AG	T R D G H S CGG GAC GGA CAT TCT	G N T L Y GGA AAT ACG CTC TAT-1.3	B17
C A S R TGT GCC AGC AG	G G L G P GGA CTG GGG CCT	S A E T L Y AGT GCA GAA ACG CTG TAT-2.3	B8
C A S S TGT GCC AGC AG	T M G T R ATG GGG ACA AGA	N T L Y AAC ACC TTG TAC-2.5	B2,B4,B5
C A S S TGT GCC AGC AG	T I S F ATA TCC TTT	Y E Q Y TAT GAA CAG TAC-2.7	B14

Figure 2.6. Junctional diversity and Jβ region usage in Vβ6$^+$ poly-18 reactive T cell hybridomas

The 3' end of the Vβ6 gene and the 5' end of the Jβ's are shown in the figure. The names of the Jβ's are given for each clone. Junction sequences flanking the Dβ regions are shown between Vβ6 and Jβ sequences.

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Chapter 3

T cell Receptor Vβ6 and Vα11 Junctional Diversity and J Region Usage in the BALB/c poly-18 T cell Response[1]

The αβ TCR recognizes processed antigen that is associated with molecules of the MHC (1,2). Crystallographic data on class I MHC offers a structural model of the Ag-MHC complex (3). Viewed from the top, there are two α-helices laying parallel to each other on top of a β-pleated sheet. The cleft created between the two α-helices is proposed to be the antigen binding cleft (4). Sequence comparison between class I and II MHC molecules suggests that class II possess an analogous structure (5). Models of TCR recognition have been generated based on the structure of MHC and on the assumption that TCR and Ig structure will be similar (6,7). The proposed positioning of the TCR over the Ag-MHC complex has the first and second hypervariable domains (encoded by the Vα and Vβ genes) over the α-helices of the MHC molecule. The third hypervariable domains (encoded by the V-D-Jβ and V-Jα junctions) lie in the middle, available for interaction with antigen. This scenario may well accurately describe a large portion of TCR recognition patterns.

1. A version of this chapter has been submitted for publication, Kilgannon P., Z. Novak, M. Sadelain, J. Ratanavongsiri, T. Dillon, B. Singh, and A. Fotedar. 1990.

While many of the antigen systems described to date might fit this model of T cell recognition (8-15), a number of notable exceptions exist. The T cell response to the hapten pABA on I-A^d or I-A^k is dominated by the use of Vα3 (16). At least 2 Jα segments and 3 Vβ genes can participate in the response. The authors suggest that Vα3 alone is largely responsible for pABA recognition. Likewise, the myoglobin response is dominated by Vβ8.2 with at least 2 Jβ's, 2.5 and 2.6 (17,18). At least 3 different Vα genes and 4 Jα regions can pair with Vβ8.2 to generate myoglobin reactivity. The T cell response to superantigens is an extreme example of one segment on one chain of the TCR dominating a T cell response. The response to an unidentified B cell antigen on I-E is associated with Vβ17a (19,20). T cell responses to minor stimulatory antigens (Mls) are associated with particular Vβ genes also. Mls-1^a is recognized by T cells expressing either Vβ6, 8.1 or 9 (21-23). Recognition of Mls-2^a is associated with expression of Vβ3 (24,25). The correlation with certain Vβ genes is so strong that expression of these self antigens has been associated with the thymic deletion of almost all T cells bearing the appropriate Vβ gene. The role of the β chain junctional diversity and the entire α chain appears to be secondary to the dominant Vβ gene in the response. A functional definition of a superantigen has emerged based on this pattern of recognition. Exogenously introduced antigen can also fall into the superantigen category. The T cell response to a number of Staphylococcal enterotoxins has been shown to follow the same pattern of response (26,27).

The T cell response to poly-18 may be an example of an antigen system that doesn't fit the conventional T cell recognition scheme. Poly-18 is a synthetic peptide antigen, a polymer of the monomeric sequence EYK(EYA)$_5$ (28). BALB/c and DBA/2 strains (H-2^d) are high responders to this antigen while H-2^b and H-2^k strains are nonresponders (29,30). Analysis of the variable gene usage in a panel of BALB/c derived poly-18/I-A^d specific T cell hybridomas revealed the dominant use of 2 V genes, Vα11(25%) and Vβ6 (50%) (Chapter 2). These are expressed in mutually exclusive groups, thus accounting for 75% of the TCR repertoire to poly-18. A broad number of other chains can associate with the dominant V genes. At least 3 Vβ's with Vα11 and 5 Vα's with Vβ6. Analysis of the junctional diversity and J region usage associated with the dominant V genes in the hybridoma analysis indicated that the amount of diversity tolerated in these areas might be quite large.

In this study, we have assessed the junctional diversity and J region usage associated with the dominant V genes in a number of poly-18 specific bulk T cell lines. The polymerase chain reaction (PCR) was used to amplify and clone Vβ6 and Vα11 junction region sequences from the mixed populations of poly-18 reactive T cells. The extent of the junctional diversity in both the Vα11 and Vβ6 repertoires is enormous. We feel that these regions of the TCR may be playing only a secondary role in conferring poly-18/I-Ad reactivity.

Material and Methods

T Cell Lines

BALB/c mice (2-3 mice per group) were immunized in the hind foot pad with 50 μg of poly-18 or $(EYA)_5$ emulsified in complete Freund's adjuvant (CFA). A cell suspension was made from the nylon wool separated popliteal lymph node cells 7-9 days after immunization. Lymph node T cells ($2\text{-}4x10^5$/ml) were cultured with irradiated (3000rad) BALB/c spleen cells ($3x10^6$/ml) and 2μM antigen in RPMI 1640 (Gibco) supplemented with 10% bovine serum, $5x10^{-5}$ M 2-mercaptoethanol, 10 mM Hepes, 2 mM glutamine and penicillin-streptomycin. T cell lines were carried in culture for 4 6 weeks as described previously (29). The author would like to acknowledge the contribution of Jana Lauzon in the generation and maintenance of the poly-18 specific T cell lines.

Proliferation Assay

Lymph node T cells lines ($2x10^5$) were cultured with irradiated (3000 rad) BALB/c spleen cells (10^6) and 20 μg poly-18 or $(EYA)_5$ in 200 μl RPMI 1640 and 10% fetal calf serum (FCS). Assay cultures were set up in duplicate or triplicate. Cultures were pulsed 3 days later with 1.0 μCi of ^{3}H-thymidine/well and harvested/counted after an additional 16 hours. Cultures were also assayed for reactivity to PPD to ensure that only poly-18 specific T cells remained in culture.

RNA Preparation

Total cellular RNA was prepared from 10^7-10^8 T cells after purification on a lympholyte M gradient (Cedar Lane Laboratories). The standard guanidium isothiocyanate and cesium chloride method was used (31). Following ultracentifugation the RNA was extracted 3X with an equal volume of phenol:chloroform (1:1) before being precipitated at -70°C in 0.1 volume NaOAc(3M) and 2 volumes ethanol (98%). This extraction and precipitation procedure was repeated one more time before the RNA was used for cDNA synthesis.

cDNA Synthesis & Polymerase Chain Reaction

Conditions for cDNA and PCR were essentially as described by Erlich (32). First strand cDNA synthesis was carried out using 1-2μg total cellular RNA, 100 pmoles random hexamers (primer), 0.25 mM dNTP's, 0.1 volume 10X PCR buffer (KCl; 0.5M, Tris-8.3; 0.2M, MgCl; 25mM, BSA; 1mg/ml) in a 20 μl volume. The RNA solution was incubated at 95°C for 1 minute then quick cooled on ice before 200 units of MuLV Reverse transcriptase (Gibco BRL) was added. The reaction was incubated at room temperature for 10 minutes followed by 37°C for an additional 60 minutes. The entire RTase reaction was used in the subsequent PCR step. Additional 10X PCR buffer and dNTP's (1 mM) were added to the RTase reaction as well as 0.25μM of each of the two primers (Vβ6 and Cβ or, Vα11 and Cα-sequences shown in Figure 3.1). The final reaction volume was 100 μl to which 4 units of Taq polymerase (Promega) was added. The reaction was overlayed with parafin oil to prevent evaporation. Temperature

cycling steps included a denaturation step (92°C; 2 min.), an annealing step (55°C; 1 min.) and and extension step (72°C; 1 min.). This cycle was repeated 35 times in an Ericomp Temperature Cycler (San Diego, Ca). A portion of the PCR reaction was run on a 2% agarose gel and the DNA band was trapped on DE81 ion exchange paper. DNA on the paper was washed in a low salt buffer (Tris pH8:10mM, EDTA;1mM, NaCl:100mM). The DNA was eluded off the paper in a high salt buffer (Tris pH8:10mM, EDTA;1mM, NaCl:1M) and precipitated in ethanol.

Cloning of PCR Products

The purified PCR product was phosphorylated with polynucleotide kinase (PNK) and blunt-ended with T4 DNA polymerase before being ligated into the SmaI site of pUC 18. Reaction conditions were essentially as described in Maniatis et al. (31). Ligation reactions were transformed into competent TB-1 *E. coli* and plated onto LB agar plates containing ampicillin and X-gal. The bacteria was incubated at 37°C overnight. Recombinant (white) colonies were picked for sequencing.

Double stranded Sequencing

Plasmid mini-preps were done on recombinant colonies. Overnight cultures were grown in 10 ml of TB,"Terrific Broth" (for 1 litre- KH_2PO_4:2.31g, K_2HPO_4:12.5g, Tryptone:12g, Yeast Extract:20g, glycerol:4ml). Bacteria were pelleted by centrifugation at 3000 rpm for 10 min. The pellet was resuspended in 250 µl glucose solution (glucose:250mM, Tris pH8:25mM, EDTA:10mM) and transferred to an

Eppindorf tube. Following the addition of 500 µl NaOH/SDS solution (NaOH:0.2M, SDS:1%) the contents of the tube were mixed by inversion for 3 min. Next, 250 µl of 5M potassium acetate was added and the tube was placed on ice for 3-5 min. The sample was centrifuged at 12,000 rpm for 3 min to pellet the debris. The supernatant was transferred to another Eppindorf tube containing 500 µl isopropanol and centrifuged for 5 minutes to precipitate the plasmid DNA. The DNA pellet was resuspended in 20 ml TE (Tris pH8:10mM, EDTA:1mM). Double stranded DNA sequencing was done according to the guidelines given in the Sequenase DNA sequencing kit (United States Biochemicals). A nested Cα and Cβ primer was used in place of the universal primer in the sequencing reactions (shown in Figure 3.1).

Results

We analysed the junctional diversity associated with each of the dominant V genes, Vβ6 and Vα11, in the BALB/c anti-poly-18 T cell response. Specific primers in the V and C regions were used to amplify and clone Vβ6 and Vα11 sequences from the RNA from bulk T cell lines reactive to poly-18 (shown in figure 3.1). While the bulk T cell lines are reactive to poly-18, we don't know the frequency of poly-18 specific T cells relative to nonreactive cells. Therefore we cannot be certain that all of the TCR sequences that we clone from the T cell lines by PCR are specific for poly-18.

The junctional region and J region usage associated with Vα11 in 2 poly-18 reactive cell lines from BALB/c (E5 and T38) are shown in fig. 3.2. The region of Vα11 from the oligonucleotide used in the amplification is shown at the top of the page. The first codon of the Cα region is shown for each sample following the Jα sequence (underlined in Fig 3.2). The amino acid sequence is included above the DNA sequence for each sample. The number of times that each clone was identified in each of the two cell lines is indicted in the columns under each cell line name. Where possible, the name of the Jα segments is given (8, 33-38). Those not identified previously in the literature are named P18.1, P18.2 and so on. Interestingly, all of the clones use a new member of the Vα11 family that was identified in the BALB/c T cell hybridomas B6 and B15 (Chapter 2). This is in contrast to the cytochrome c/I-E^k response which uses Vα11.1 and 11.2 (8). A notable difference between the Vα11 family members is the presence of an extra codon in the Vα11 seen here. In 34 clones

analysed here, 16 different Jα regions are identified. All but one of the J regions are found in only one rearrangement pattern with Vα11. We feel that this is actually an underestimate of the Jα usage in the poly-18 response given the number of clones that were only detected once in the analysis and the limited overlap seen between the two lines.

The situation with the Vβ6 portion of the repertoire is even more striking. We isolated Vβ6 sequences from 6 different BALB/c poly-18 reactive T cell lines (Fig. 3.3a-f). The 3' portion of the Vβ6 gene and 5' portion of the Jβ's are shown in the figure. Amino acid sequences (single letter code) are shown above the nucleotide sequences. The number of times that each clone was identified is indicated on the right side of the figure. A total of 145 sequences were generated, yielding 55 different Vβ6 clones. The limited overlap between the different cell lines is demonstrated in Fig 3.4. This shows the junctional amino acid sequences associated with each of the Jβ's and the frequency that a given clone was identified in each cell line. The lack of overlap between the different T cell lines may indicate that the total potential Vβ6 repertoire to poly-18 is very large. Eleven of the 12 possible Jβ regions are used in the response. Within individual Jβ's, numerous junctional sequences can be found. For example, there are 11 different junction sequences observed with Jβ2.7, and 8 in each of Jβ2.5 and 2.3 (see fig. 3.4). Other Jβ regions are seen in only one configuration (Jβ1.6 and 2.2). The overlap between the 6 cell lines is minimal. Only 4 of the 55 clones are seen in more that one cell line (indicated with an * in the

figures 3.3a-f). This leads us to speculate that the true BALB/c anti-poly-18 Vβ6 repertoire is enormous.

Discussion

Our initial analysis of the BALB/c T cell receptor repertoire to poly-18 identified the predominant use of Vα11 (25%) and Vβ6 (50%) (Chapter 2). The number of other chains that could associate with the dominant V gene chains was quite large considering the sample sizes. Also, the junctional diversity and J region usage connected with the dominant V genes in the hybridomas seemed extensive too. This led us to speculate that the poly-18 reactivity of T cells expressing either Vα11 or Vβ6 might be conferred largely by the dominant V genes themselves. We have extended the analysis of the junctional diversity and J region usage associated with these two V genes by going back to bulk T cell lines. The polymerase chain reaction allowed us to specifically amplify and clone Vα11 and Vβ6 junction sequences. This meant that we could effectively analyse larger sample sizes and more cell lines. While the junctional diversity is seemingly larger in Vβ6, this may be a result of diversity created by two rearrangement events and the D regions ability to be read in all three reading frames (39). No apparent selection for particular amino acids can be detected in either Vβ6 or Vα11 junctions. The results generated by this analysis have confirmed the trend observed in the hybridoma analysis. Simply stated, the amount of junctional diversity and J region usage associated with these two V genes in the poly-18 response is enormous.

What are the implications of this range of diversity? The current model of T cell recognition places the third hypervariable domain, those sequences encoded by the junction regions, over the

peptide in the antigen cleft (6,7). Is it conceivable that the 55 different Vβ6 junction/Jβ sequences identified in this analysis are responsible for conferring specificity to poly-18? Similarly, what is the role of the 16 Vα11-junction/Jα sequences in conferring poly-18 reactivity? It seems that rather than conferring poly-18 reactivity, these regions are tolerated by receptors that already have an affinity for the antigen, conferred largely by the dominant V genes. Their presence obviously has some effect on the interaction with poly-18/I-A^d, or poly-18 would be a superantigen, stimulating almost all Vβ6 and Vα11 bearing T cells.

A distinction needs to be made between overall reactivity to an antigen and the fine specificity profile. Numerous systems have shown the importance of junction sequences in the determination of fine specificity (40,41). In cytochrome c responses there is a selection for certain amino acids at position 100, which is in the junction region (42). In Vβ3, asparagine is selected, and in Vβ1 or Vβ16, aspartic acid is selected. Mutagenesis of this site alters or abolishes the response (43). Other systems, like myelin basic protein (11-13), TNP/H-$2K^b$ (14,15), and λ repressor (41), note a very limited number of junction/J regions used. In these systems, the strong selective pressure for all elements of the TCR is usually evident, implying the involvement of all elements in conferring specificity for that antigen/MHC complex. This is in contrast to superantigen recognition (19-27) and to a lesser degree, the responses to poly-18, pABA (16) and myoglobin (17,18). In these responses, greater flexibility in some components of the TCR is evident from the number of alternate elements used. In the pABA response, Vα3

predominates with at least 2 Jα and 3 Vβ genes shown to associate. A number of lines of evidence suggests that Vα3 confers general reactivity to pABA on as many as 3 different MHC molecules. Superantigen responses represent the most extreme case, in that the dominance of the Vβ gene segment is such that almost any Jβ or α chain can be present and not interfere with reactivity. However, the strong association between Vβ genes and superantigen recognition is not absolute, as discussed in Chapter 4 of this thesis. Thus we must refer to the relative influence of different components of the T cell receptor on antigen reactivity.

Poly-18 recognition by T cells that express either Vα11 or Vβ6 can accommodate numerous other chains paired with the dominant V gene chains (Chapter 2). In addition, the amount of junctional diversity and J region usage found associated with the dominant V genes indicates that these regions play a secondary role in overall poly-18 reactivity. This is to be distinguished from their influence on fine specificity. The microheterogeneity of antigen fine specificity response patterns seen in the poly-18 system is extensive (45), as might be predicted from the analysis of the TCR genes involved in the response. Many selective pressures operate on the immune system. Antigen selects TCR's capable of recognizing it from among the available peripheral T cell repertoire. If the primary determinant of reactivity is conferred by a particular V gene, then the selective pressure to be associated with a few J regions or limit junctional diversity will be reduced. Consequently, a large number of TCR's bearing that V gene in conjunction with a variety of other TCR components will be selected to participate in the response. The

relative influence of those other components will determine the number of different combinations that are permissible. In the case of poly-18 reactivity by TCR's using either Vβ6 or Vα11, the broad range of other chains and junction regions indicates a relatively minor role in conferring overall reactivity to poly-18. The simple repeating nature of the antigen may be responsible for the nature of some T cell responses to it. A degree of flexibility in the way that poly-18 is bound by I-A has been suggested (46). The underlying complexity of T cell recognition is evident in the variety of TCR gene usage patterns seen in responses to different antigens.

Cα(5')	antisense	5'GAGGG	TGCTG	TCCTG	AGACC	GAGG3'
Cα(3')	antisense	5'GGTTT	TCGGC	ACATT	GATTT	GGGA3'
Vα11	sense	5'CTACT	TGGCT	TCAGG	AACAA	AGGA3'
Cβ(5')	antisense	5'CAAGG	AGACC	TTGGG	TGGAG	TCAC3'
Cβ(3')	antisense	5'TCTCT	GCTTT	TGATG	GCTCA	AACA3'
Vβ6	sense	5'GGCGA	TCTAT	CTGAA	GGCTA	TGAT3'

Figure 3.1. Oligonucleotides used for PCR amplification and sequencing of Vα11 and Vβ6 junction regions.

```
 Y  L  A  S  G  T  K  E  N  G  R  L  K  S  A  F  D  S  K  E
CTAC TTG GCT TCA GGA ACA AAG GAG AAT GGG AGG CTA AAG TCA GCA TTT GAT TCT AAG GAG

 R  R  Y  S  T  L  H  I  R  D  A  Q  L  E  D  S  G  T  Y  F
CGG CGC TAC AGC ACC CTG CAC ATC AGG GAT GCC CAG CTG GAG GAC TCA GGC ACT TAC TTC
```

Vα11	Jα	Name	T38	E5
C A A	Y T G A N T G K L T			
TGT GCT GC	C TAC ACT GGA GCT AAC ACT GGA AGG CTC ACG			
F G H G N I L R V H P N I				
TTT GGA CAC GGC ACC ATC CTT AGG GTC CAT CCA A AC ATC		C5	4	-
C A A	E A L Q T G F A S A L T			
TGT GCT GC	T GAG GCA TTG CAG ACA GGC TTT GCA AGT GCG CTG ACA			
F G S G T K V I V L P Y I				
TTT GGA TCT GGC ACA AAA GTC ATT GTT CTA CCA T AC ATC		PHDS58	4	-
C A A	S S N T D K V V			
TGT GCT GC	G TCT TCC AAT ACC GAC AAA GTC GTC			
F G T G T R L Q V S P N I				
TTT GGA ACA GGG ACC AGA TTA CAA GTC TCA CCA A AC ATC		P-18.1	1	-
C A A	V T G S G G K L T			
TGT GCT GC	C GTT ACT GGC AGT GGT GGA AAA CTC ACT			
L G T G T R L Q V N L D I				
TTG GGG ACT GGA ACA AGA CTT CAG GTC AAC CTT G AC ATC		P-18.2	1	-
C A A	L Y A N K M I			
TGT GCT GC	C TTA TAT GCA AAC AAG ATG ATC			
F G L G T I L R V R P H I				
TTT GGC TTG GGA ACC ATT TTG AGA GTC AGA CCT C AC ATC		P-18.3	1	-
C A	G G G - N N K L T			
TGT GCT G	GG GGC GGA GnC AAT AAT AAG CTG ACT			
F G Q G T V L S V I P D I				
TTT GGT CAA GGA ACC GTT CTG AGT GTT ATA CCA G AC ATC		TA2B4	1	-
C A A	G N N N R I F			
TGT GCT GC	T GGT AAC AAT AAC AGA ATC TTC			
F G D G T Q L V V K P N I				
TTT GGT GAT GGG ACG CAG CTG GTG GTG AAG CCC A AC ATC		MD13	4	1
C A A	H Q G G R A L I			
TGT GCT GC	C CAC CAG GGA GGC AGA GCT CTG ATA			
F G T G T T V S V S P N I				
TTT GGA ACA GGA ACC ACG GTA TCA GTC AGC CCC A AC ATC		P-18.4	1	2
C A A	Y R G S A L G R L H			
TGT GCT GC	C TAT AGA GGT TCA GCC TTA GGG AGG CTG CAT			
F G A G T Q L I V I P D I				
TTT GGA GCT GGG ACT CAG CTG ATT GTC ATA CCT G AC ATC		LB2	1	4
C A A	G N Q G G S A K L I			
TGT GCT GC	T GGG AAT CAA GGA GGG TCT GCG AAG CTC ATC			
F G E G T K L T V S S Y I				
TTT GGG GAG GGG ACA AAG CTG ACA GTG AGC TCA T AC ATC		TA27	-	2

C	A	A						C	S	G	G	S	N	Y	K	L	T							
TGT	GCT	GC					T	TGC	AGT	GGA	GGC	AGC	AAT	TAC	AAA	CTG	ACA							
			F	G	K	G	T	L	L	T	V	T	P			N	I							
			TTT	GGG	AAA	GGA	ACT	CTC	TTA	ACT	GTG	ACT	CCA	A		AC	ATC	3DT	-	2				
C	A	A						G	S	S	G	S	F	N	K	L	T							
TGT	GCT	GC					G	GGA	AGT	TCT	GGT	AGC	TTC	AAT	AAG	TTG	ACC							
			F	G	A	G	T	R	L	A	V	C	P			Y	I							
			TTT	GGA	GCA	GGG	ACC	AGA	CTG	GCT	GTG	TGC	CCA	T		AC	ATC	P-18.5	-	2				
C	A	A				E	D	H	P	G	A	N	T	G	K	L	T							
TGT	GCT	GC		T	GAG	GAC	CAC	CCT	GGA	GCT	AAC	ACT	GGA	AAG	CTC	ACG								
			F	G	H	G	T	I	L	R	V	H	P			N	I							
			TTT	GGA	CAC	GGC	ACC	ATC	CTT	AGG	GTC	CAT	CCA	A		AC	ATC	C5	-	1				
C	A	A						G	A	N	T	G	Y	Q	N	F	Y							
TGT	GCT	GC					T	GGT	GCC	AAC	ACG	GGT	TAC	CAG	AAC	TTC	TAT							
			F	G	K	G	T	S	L	T	V	I	P			N	I							
			TTT	GGG	AAA	GGA	ACA	AGT	TTG	ACT	GTC	ATT	CCA	A		AC	ATC	TA65	-	1				
C	A	A						E	A	G	G	T	G	S	K	L	S							
TGT	GCT	GC					T	GAG	GCG	GGG	GGC	ACT	GGG	TCT	AAG	CTG	TCA							
			F	G	K	G	A	K	L	T	V	S	P			D	I							
			TTT	GGG	AAG	GGG	GCA	AAG	CTC	ACA	GTG	AGT	CCA	G		AC	ATC	TA1	-	1				
C	A	A						E	A	L	G	N	T	R	K	L	I							
TGT	GCT	GC					T	GAG	GCC	TTA	GGC	AAT	ACT	AGA	AAA	CTC	ATC							
			F	G	L	G	-	T	L	Q	V	Q	P			D	I							
			TTT	GGG	CTG	GGG	NCA	ACT	TTA	CAA	GTG	CAA	CCA	G		AC	ATC	TA57	-	1				
																		TOTAL		17	17			

Figure 3.2. Junctional diversity and J region usage in Vα11 sequences from BALB/c poly-18 specific T cell lines, T38 & E5.

Single letter amino acid code is given above each codon. Jα regions previously described are given original designation, others are named P-18.X (8, 33-38). The sequence that is underlined at the end of each Jα, is the first 5 nucleotides of the Cα region. The region of Vα11 that is underlined is one of the oligonucleotides used in the PCR to amplify this region. All clones used the same member of the Vα11 family. The extra codon (GCC) found in this member of the Vα11 family is highlighted in the figure.

Vβ6	Dβ	Jβ	#
C A S S TGT GCC AGC AG	C	M G Q ATG GGA CAA	N S D Y T F G AAC TCC GAC TAC ACC TTC GGC-1.2	2
C A S S TGT GCC AGC AG	T	M G T G ATG GGG ACA GGG	S G N T L Y F G AGT GGA AAT ACG CTC TAT TTT GGA-1.3	6
C A S S TGT GCC AGC AG	T	M G Q L S ATG GGA CAA CTT TCT	G N T L Y F G GGA AAT ACG CTC TAT TTT GGA-1.3	2
C A S S TGT GCC AGC AG	T	I S G Y S ATA TCA GGT TAT TCT	G N T L Y F G GGA AAT ACG CTC TAT TTT GGA-1.3	1
C A S S TGT GCC AGC AG	T	S G Q G S AGT GGA CAG GGG TCT	G N T L Y F G GGA AAT ACG CTC TAT TTT GGA-1.3	1
C A S S TGT GCC AGC AG	T	L R G L CTC AGG GGG CTT	S N E R L F F G TCC AAC GAA AGA TTA TTT TTC GGT-1.4	1
C A S S TGT GCC AGC AG	T	A G H GCG GGC CAC	N E R L F F G AAC GAA AGA TTA TTT TTC GGT-1.4	1
C A S S TGT GCC AGC AG	T	R D AGA GAC	S Y N S P L Y F A AGC TAT AAT TCG CCC CTC TAC TTT GCG-1.6	2
C A S S TGT GCC AGC AG	T	I A T R G ATA GCT ACC AGG GGA	A E Q F F G GCT GAG CAG TTC TTC GGA-2.1	2
C A S S TGT GCC AGC AG	T	I R G V ATT AGG GGG GTA	Y A E Q F F G TAT GCT GAG CAG TTC TTC GGA-2.1	1
C A S S TGT GCC AGC AG	T	R T G G CGG ACA GGG GGC	T G Q L Y F G ACC GGG CAG CTC TAC TTT GGT-2.2	1
C A S R TGT GCC AGC AG	G	G L G P GGA CTG GGG CCT	S A E T L Y F G AGT GCA GAA ACG CTG TAT TTT GGC-2.3	4
C A S S TGT GCC AGC AG	T	L R T G CTC CGG ACA GGG	E T L Y F G GAA ACG CTG TAT TTT GGC-2.3	1
C A S TGT GCC AGC A	AA	K Q G A T CAG GGG GCG ACA	E T L Y F G GAA ACG CTG TAT TTT GGC-2.3	1
C A S S TGT GCC AGC AG	T	I R ATA CGG	D T Q Y F G GAC ACC CAG TAC TTT GGG-2.5	1
C A S S TGT GCC AGC AG	T	R L G A I GCA CTG GGG GCC ATC	Y E Q Y F G TAT GAA CAG TAC TTC GGT-2.7	2
C A S S TGT GCC AGC AG	T	I T G G L ATC ACA GGG GGC CTC	S Y E Q Y F G TCC TAT GAA CAG TAC TTC GGT-2.7	1
				30

Figure 3.3a. Junctional diversity and J region usage in Vβ6 sequences from BALB/c poly-18 specific T cell line, T38.

Single letter amino acid code is given above each codon. Only the 3' end of Vβ6 and 5' ends of the Jβ's are shown in the figure. The number of times that a clone was isolated is shown on the right hand column.

Vβ6	Dβ	Jβ	#
C A S S TGT GCC AGC AG	M G V G T ATG GGG GTG GGC	S G N T L Y F G TCT GGA AAT ACG CTC TAT TTT GGA-1.3	1
C A S S TGT GCC AGC AG	R T V T CGG ACA GTT	S N E R L F F G TCC AAC GAA AGA TTA TTT TTC GGT-1.4	2*
C A S S TGT GCC AGC AG	R G S C CGG GGG TCC	Y A E Q F F G TAT GCT GAG CAG TTC TTC GGA-2.1	4
C A S S TGT GCC AGC AG	L R G C CTC CGG GGG	N Y A E Q F F G AAC TAT GCT GAG CAG TTC TTC GGA-2.1	1
C A S TGT GCC AGC A	T R T G G CC CGG ACT GGG GGT	N Y A E Q F F G AAC TAT GCT GAG CAG TTC TTC GGA-2.1	1
C A S S TGT GCC AGC AG	I A H R V T ATA GCC CAC AGG GTC	A E T L Y F G GCA GAA ACG CTG TAT TTT GGC-2.3	3
C A S S TGT GCC AGC AG	R G G P T CGG GGG GGC CCT	S Q N T L Y F G AGT CAA AAC ACC TTG TAC TTT GGT-2.4	4
C A S S TGT GCC AGC AG	M T G G L S T ATG ACT GGG GGG CTG TCA	N T L Y F G AAC ACC TTG TAC TTT GGT-2.4	1
C A S R TGT GCC AGT AG	D R G GAC AGG	D T Q Y F G GAC ACC CAG TAC TTT GGG-2.5	8*
C A TGT GCC	S R D R TCC CGG GAC AGG	D T Q Y F G GAC ACC CAG TAC TTT GGG-2.5	1
C A S S TGT GCC AGC AG	I G T R N T ATT GGG ACT CGG AAC	T Q Y F G ACC CAG TAC TTT GGG-2.5	1
C A S R TGT GCC AGC AG	R D R D G CGG GAC AGG GAC	E Q Y F G GAA CAG TAC TTC GGT -2.7	1
C A S TGT GCC AGC	K Q G P AAA CAG GGT CCC	S Y E Q Y F G TCC TAT GAA CAG TAC TTC GGT-2.7	2*
C A S S TGT GCC AGC AG	P G R T CCG GGA CGG	Y E Q Y F G TAT GAA CAG TAC TTC GGT-2.7	1
C A S S TGT GCC AGC AG	I G G G R T ATT GGG GGG GGC GCG	S Y E Q Y F G TCC TAT GAA CAG TAC TTC GGT-2.7	1
			32

* Denotes clones seen in other BALB/c anti- poly-18 cell lines.

Figure 3.3b. Junctional diversity and J region usage in Vβ6 sequences from BALB/c poly-18 specific T cell line, E5.

Single letter amino acid code is given above each codon. Only the 3' end of Vβ6 and 5' ends of the Jβ's are shown in the figure. The number of times that a clone was isolated is shown on the right hand column.

Vβ6	Dβ	Jβ	#
C A S TGT GCC AGC A	K D R E R AG GAC AGG GAA AGG	V F F G GTC TTC TTT GGT-1.1	6
C A S S TGT GCC AGC AG	R T S Y C CGA ACA TCC TAC	T E V F F G ACA GAA GTC TTC TTT GGT-1.1	1
C A S S TGT GCC AGC AG	I G Q T ATA GGA CAA	N S D Y T F G AAC TCC GAC TAC ACC TTC GGC-1.2	4
C A S S TGT GCC AGC AG	R T L T CGG ACA CTT	S N E R L F F G TCC AAC GAA AGA TTA TTT TTC GGT-1.4	1*
C A S S TGT GCC AGC AG	I R D R A T ATC CGG GAC AGG GCT	A E T L Y F G GCA GAA ACG CTG TAT TTT GGC-2.3	2
C A S R TGT GCC AGC AG	D R G P G GAC AGG GGT CCT	S A E T L Y F G AGT GCA GAA ACG CTG TAT TTT GGC-2.3	2
C A S TGT GCC AGC A	T R T A CC CGG ACA GCT	S Q N T L Y F G AGT CAA AAC ACC TTG TAC TTT GGT-2.4	2
C A S R TGT GCC AGC AG	N R A AAC AGG	D T Q Y F G GAC ACC CAG TAC TTT GGG-2.5	1
C A S R TGT GCC AGC AG	D R E G GAC AGG GAG	E Q Y F G GAA CAG TAC TTC GGT-2.7	5
C A S S TGT GCC AGC AG	I K A T ATA AAG GCC	S Y E Q Y F G TCC TAT GAA CAG TAC TTC GGT-2.7	1

* Denotes clones seen in other BALB/c anti- poly-18 cell lines. 25

Figure 3.3c. Junctional diversity and J region usage in Vβ6 sequences from BALB/c poly-18 specific T cell line, E.E.

Single letter amino acid code is given above each codon. Only the 3' end of Vβ6 and 5' ends of the Jβ's are shown in the figure. The number of times that a clone was isolated is shown on the right hand column.

Vβ6	Dβ	Jβ	#
C A S S	R T L	S N E R L F F G	
TGT GCC AGC AG	T CGG ACA CTT	TCC AAC GAA AGA TTA TTT TTC GGT-1.4	3*
C A S S	R T V	S N E R L F F G	
TGT GCC AGC AG	T CGG ACA GTT	TCC AAC GAA AGA TTA TTT TTC GGT-1.4	1*
C A S S	P L G G G R	D T Q Y F G	
TGT GCC AGC AG	T CCA CTG GGG GGG AGC	GAC ACC CAG TAC TTT GGG-2.5	6
C A S	K Q G P	S Y E Q Y F G	
TGT GCC AGC A	AA CAG GGT CCC	TCC TAT GAA CAG TAC TTC GGT-2.7	6*
			16

* Denotes clones seen in other BALB/c anti- poly-18 cell lines.

Figure 3.3d. Junctional diversity and J region usage in Vβ6 sequences from BALB/c poly-18 specific T cell line, E.P.

Single letter amino acid code is given above each codon. Only the 3' end of Vβ6 and 5' ends of the Jβ's are shown in the figure. The number of times that a clone was isolated is shown on the right hand column.

Vβ6	Dβ	Jβ	#
C A S S TGT GCC AGC AG	A G T G R T GCC GGG ACA GGG AGG	T L Y F G ACG CTC TAT TTT GGA-1.3	2
C A S S TGT GCC AGC AG	R T L T CGG ACA CTT	S N E R L F F G TCC AAC GAA AGA TTA TTT TTC GGT-1.4	2*
C A S R TGT GCC AGC AG	N R - Y A AAC AGG G-C TAT	S N E R L F F G TCC AAC GAA AGA TTA TTT TTC GGT-1.4	1
C A S S TGT GCC AGC AG	P G Q Y C CCG GGA CAA TAT	S N E R L F F G TCC AAC GAA AGA TTA TTT TTC GGT-1.4	1
C A S S TGT GCC AGC AG	H T G G R T CAT ACA GGG GGC CGA	D T Q Y F G GAC ACC CAG TAC TTT GGG-2.5	1
C A S TGT GCC AGC A	K D R AG GAC AGG	D T Q Y F G GAC ACC CAG TAC TTT GGG-2.5	5
C A S TGT GCC AGC A	K D R D AA GAC AGA GAT	E Q Y F G GAA CAG TAC TTC GGT-2.7	4
C A S S TGT GCC AGC AG	P - L G T CCC -GA CTG GGG	Y E Q Y F G TAT GAA CAG TAC TTC GGT-2.7	1
			17

* Denotes clones seen in other BALB/c anti- poly-18 cell lines.

Figure 3.3e. Junctional diversity and J region usage in Vβ6 sequences from BALB/c poly-18 specific T cell line, P.E.

Single letter amino acid code is given above each codon. Only the 3' end of Vβ6 and 5' ends of the Jβ's are shown in the figure. The number of times that a clone was isolated is shown on the right hand column.

Vβ6	Dβ	Jβ	#
C A S R TGT GCC AGC AG	A T G S A ACA GGA TCA	N S D Y T F G AAC TCC GAC TAC ACC TTC GGC-1.2	3
C A S S TGT GCC AGC AG	R T L T CGG ACA CTT	S N E R L F F G TCC AAC GAA AGA TTA TTT TTC GGT-1.4	5*
C A S R TGT GCC AGC AG	G T F G GGG ACA TTT	S N E R L F F G TCC AAC GAA AGA TTA TTT TTC GGT-1.4	1
C A S S TGT GCC AGC AG	R G L R T AGG GGA CTG AGG	A E T L Y F G GCA GAA ACG CTG TAT TTT GGC-2.3	1
C A S S TGT GCC AGC AG	L R D R R T CTC CGG GAC AGG CGT	A E T L Y F G GCA GAA ACG CTG TAT TTT GGC-2.3	9
C A S R TGT GCC AGC AG	D R G GAC AGG	D T Q Y F G GAC ACC CAG TAC TTT GGG-2.5	2*
C A S S TGT GCC AGC AG	I A V M T ATA GCT GTC ATG	Y E Q Y F G TAT GAA CAG TAC TTC GGT-2.7	2
C A S TGT GCC AGC A	K Q G P AA CAG GGT CCC	S Y E Q Y F G TCC TAT GAA CAG TAC TTC GGT-2.7	2*
			25

* Denotes clones seen in other BALB/c anti- poly-18 cell lines.

Figure 3.3f. Junctional diversity and J region usage in Vβ6 sequences from BALB/c poly-18 specific T cell line, P.P.

Single letter amino acid code is given above each codon. Only the 3' end of Vβ6 and 5' ends of the Jβ's are shown in the figure. The number of times that a clone was isolated is shown on the right hand column.

BALB/c Poly-18 T cell Lines

Vβ6	Dβ	Jβ1.1	E5	T38	E.E	E.P	P.E	P.P
CAS	KDRER	V..	-	-	4/25	-	-	-
CAS	SRTSY	TEV..	-	-	1/25	-	-	-

Vβ6	Dβ	Jβ1.2	E5	T38	E.E	E.P	P.E	P.P
CAS	SMGQ	NSD..	-	2/30	-	-	-	-
CAS	SIGQ	NSD..	-	-	4/25	-	-	-
CAS	RTGS	NSD..	-	-	-	-	-	3/25

Vβ6	Dβ	Jβ1.3	E5	T38	E.E	E.P	P.E	P.P
CAS	SMGVG	SGNT..	1/32	-	-	-	-	-
CAS	SMGTG	SGNT..	-	6/30	-	-	-	-
CAS	SMGQLS	GNT..	-	2/30	-	-	-	-
CAS	SISGYS	GNT..	-	1/30	-	-	-	-
CAS	SSGQGS	GNT..	-	1/30	-	-	-	-
CAS	SAGTGR	T..	-	-	-	-	2/17	-

Vβ6	Dβ	Jβ1.4	E5	T38	E.E	E.P	P.E	P.P
CAS	SRTV	SNER..	2/32	-	-	1/16	-	-
CAS	SRTL	SNER..	-	-	1/25	3/16	2/17	5/25
CAS	SLRGL	SNER..	-	1/30	-	-	-	-
CAS	SAGH	NER..	-	1/30	-	-	-	-
CAS	RNRxY	SNER..	-	-	-	-	1/17	-
CAS	SPGQY	SNER..	-	-	-	-	1/17	-
CAS	RGTF	SNER..	-	-	-	-	-	1/25

Vβ6	Dβ	Jβ1.6	E5	T38	E.E	E.P	P.E	P.P
CAS	SRD	SYNS	-	2/30	-	-	-	-

Vβ6	Dβ	Jβ2.1	E5	T38	E.E	E.P	P.E	P.P
CAS	SRGS	YA..	4/32	-	-	-	-	-
CAS	SLRG	NYA..	1/32	-	-	-	-	-
CAS	TRTGG	NYA..	1/32	-	-	-	-	-
CAS	SIATRG	A..	-	2/30	-	-	-	-
CAS	SIRGV	YA..	-	1/30	-	-	-	-

Vβ6	Dβ	Jβ2.2	E5	T38	E.E	E.P	P.E	P.P
CAS	SRTGG	TGQ..	-	1/30	-	-	-	-

Vβ6	Dβ	Jβ2.3	E5	T38	E.E	E.P	P.E	P.P
CAS	SIAHRV	AE..	3/32	-	-	-	-	-
CAS	RGLGP	SAE..	-	4/30	-	-	-	-
CAS	SLRTG	E..	-	1/30	-	-	-	-
CAS	KQGAT	E..	-	1/30	-	-	-	-
CAS	SIRDRA	AE..	-	-	2/25	-	-	-
CAS	RDRGP	SAE..	-	-	2/25	-	-	-
CAS	SRGLR	AE..	-	-	-	-	-	1/25
CAS	SLRDRR	AE..	-	-	-	-	-	9/25

Vβ6	Dβ	Jβ2.4	E5	T38	E.E	E.P	P.E	P.P
CAS	SRGGP	SQN..	4/32	-	-	-	-	-
CAS	SMTGGLS	N..	1/32	-	-	-	-	-
CAS	TRTA	SQN..	-	-	2/25	-	-	-

Vβ6	Dβ	Jβ2.5	E5	T38	E.E	E.P	P.E	P.P
CAS	RDR	DTQ..	8/32	-	-	-	-	2/25
CA	SRDR	DTQ..	1/32	-	-	-	-	-
CAS	SIGTRN	TQ..	1/32	-	-	-	-	-
CAS	SIR	DTQ..	-	1/30	-	-	-	-
CAS	RNR	DTQ..	-	-	1/25	-	-	-
CAS	SPLGGR	DTQ..	-	-	-	6/16	-	-
CAS	KDR	DTQ..	-	-	-	-	5/17	-
CAS	SHTGGR	DTQ..	-	-	-	-	1/17	-

Vβ6	Dβ	Jβ2.7	E5	T38	E.E	E.P	P.E	P.P
CAS	RRDRD	E..	1/32	-	-	-	-	-
CAS	KQGP	SYE..	2/32	-	-	6/16	-	2/25
CAS	SPGR	YE..	1/32	-	-	-	-	-
CAS	SIGGGR	SYE..	1/32	-	-	-	-	-
CAS	SRLGAI	YE..	-	2/30	-	-	-	-
CAS	SITGGL	SYE..	-	1/30	-	-	-	-
CAS	RDRE	E..	-	-	5/25	-	-	-
CAS	SIKA	SYE..	-	-	1/25	-	-	-
CAS	KDRD	E..	-	-	-	-	4/17	-
CAS	SPxLG	YE..	-	-	-	-	1/17	-
CAS	SIAVM	YE..	-	-	-	-	-	2/25

Figure 3.4. Junctional diversity and J region usage associated with Vβ6 in six BALB/c poly-18 specific T cell lines.

Amino acids in the junction regions for each Jβ are shown. An "x" is put in places where amino acid determination was not possible due to sequencing ambiguities. The frequency that each clone was seen in the six poly-18 specific T cell lines (E5, T38, E.E, E.P, P.E, P.P) is given in the columns below each cell line name.

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Chapter 4

Antigen specific Vβ6 T cells not deleted in DBA/2 mice: Vβ6 junctional diversity and J region usage associated with the inability to recognize Mls-1^a [1]

The learning of self restriction and the acquisition of self tolerance are central to the generation of a functional peripheral T cell repertoire (reviewed in 1). A number of systems have been used effectively to demonstrate negative selection, i.e., the deletion of self reactive T cells during development. Some of these systems make use of the observation that specific Vβ's appear to confer reactivity to certain strong antigens, now refered to as "superantigens". For example, reactivity to class II MHC I-E antigens plus an undefined B cell antigen is associated with Vβ17a $^+$ T cells (2,3). The Mls (minor lymphocyte stimulatory) antigens also behave in this manner with Vβ6, Vβ8.1 and Vβ9 conferring reactivity to Mls-1^a (4-7), while Mls-2^a is recognized by TCR's bearing Vβ3 (8,9). Mice bearing Mls-1^a or other self superantigens are known to delete thymocytes that express Vβ genes that correlate with reactivity. The generation of TCR V region specific monoclonal antibodies (MAb's) allowed for the demonstration of thymic deletion of T cells bearing certain Vβ's in mouse strains expressing these self antigens. Similarly, a number of staphylococcal enterotoxins activate T cells

1. A version of this chapter has been submitted for publication, Kilgannon, P., Z. Novak, D. McNeil, J. Lauzon, B. Singh, and A. Fotedar. 1990.

based on Vβ gene usage (10,11). These reactivities are unique in that the role of the TCRα chain and the junctional diversity in the β chain appear to have little influence on the interaction with antigen. As in any biological system, there are exceptions to the rule. Recognition of these antigens may not always rely entirely on the Vβ region alone. Characterization of the exceptions can give an insight into the limits and parameters of T cell recognition in these model systems.

This paper addresses the potential role of the other elements in the TCR, besides the Vβ6 region, that may affect Mls-1^a recognition and clonal deletion. Our initial analysis involved the characterization of poly-18 reactive T cell hybridomas from BALB/c mice. Poly-18 is a synthetic peptide antigen composed of the repeating monomer, $EYA(EYK)_5$ (12). Response to poly-18 is under Ir gene control, with H-2^d strains (BALB/c and DBA/2) as high responders and H-2^b and H-2^k as nonresponders (13). Analysis of the BALB/c T cell repertoire to this antigen demonstrated a 50% usage of Vβ6 (Chapter 2). Interestingly, these Vβ6+ cells used a large number of different TCRα chains in the poly-18 response. Also, the junctional diversity in the Vβ6 chains was enormous, using 11 of the 12 Jβ's (Chapter 3). The Mls-1^a reactivity of 8 poly-18 specific Vβ6$^+$ T cell hybridomas from BALB/c was assessed. A number of these Vβ6 hybridomas failed to respond to Mls-1^a. The polymerase chain reaction was used to amplify and clone junctional sequences in all 8 poly-18 reactive Vβ6$^+$ hybridomas (Chapter 2). The relationship between TCR gene usage and the ability of these Vβ6$^+$ T cell hybridomas to recognize Mls-1^a is discussed.

In addition, we set out to address the question of whether deletion of Vβ6 cells was complete by purposefully looking for Vβ6$^+$ T cells in the periphery of DBA/2 mice (Mls-1a). These mice are known to delete Vβ6 bearing T cells during thymic maturation (4). This is in contrast to BALB/c mice which are Mls-1^b and consequently do not delete their Vβ6 compartment. However, simply finding Vβ6$^+$ T cells in the periphery of DBA/2 mice would not address the immunological competence of such cells. Therefore we attempted to amplify Vβ6$^+$ cells by immunizing with poly-18. The features of the poly-18 system led us to speculate that this might be an appropriate antigen to amplify rare Vβ6 bearing cells that escaped deletion in the DBA/2 thymus. Also, this would allow us to work with immunologically competent cells, removing the chance of isolating anergic clones like those described for the transgenic mice expressing Vβ8.1 (14). The polymerase chain reaction (PCR) was used to specifically amplify and clone Vβ6 sequences from poly-18 reactive bulk T cell lines derived from DBA/2 mice. We have assessed the Vβ6 junctional diversity and J region usage associated with the inability to recognize Mls-1^a.

Methods and Materials

Mice

All DBA/2 mice were obtained from The Jackson Laboratory, Bar Harbor, Me.

Antigen

Poly-18, $[EYK(EYA)_5]_n$ was prepared by the classical fragment condensation as previously described (15). Synthetic peptide antigen (EYA)5 was made by the Merrifield solid phase peptide synthesis method (16). Peptides were dissolved in saline and the pH was adjusted to 7.2 with 0.1N NaOH. Prior to use it was filter sterilized through a 0.22 Um filter. Amino acid abbreviations: K; lysine, E; glutamic acid, Y; tyrosine, A; alanine.

T Cell Lines

DBA/2 mice (2-3 mice per group) were immunized in the hind foot pad with 50 μg of poly-18 or EYA_5 emulsified in complete Freund's adjuvant (CFA). A cell suspension was made from the nylon wool separated popliteal lymph node cells 7-9 days after immunization. Lymph node T cells ($2\text{-}4x10^5$/ml) were cultured with irradiated (3000rad) DBA/2 spleen cells ($3x10^6$/ml) and 2μM antigen in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum, $5x10^{-5}$ M 2-mercaptoethanol, 10 mM Hepes, 2 mM glutamine and penicillin-streptomycin. T cell lines were carried in culture for 4-6 weeks as described previously (13). The author gratefully

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Proliferation Assay

Lymph node T cells ($2x10^5$) were cultured with irradiated (3000 rad) DBA/2 spleen cells (10^6) and 20 µg poly-18 in 200 µl RPMI 1640 and 10% fetal calf serum (FCS). Assay cultures were set up in duplicate or triplicate. Cultures were pulsed 3 days later with 1.0 µCi of ^{3}H-thymidine/well and harvested/counted after an additional 16 hours.

RNA Preparation

Total cellular RNA was prepared from 10^7-10^8 T cells after purification on a lympholyte M gradient (Cedar Lane Laboratories). The standard guanidium isothiocyanate and cesium chloride method was used (17). The RNA was extracted 3X with an equal volume of phenol:chloroform (1:1) before being precipitated at -70°C in 0.1 volume NaOAc(3 M) and 2 volumes ethanol (98%). This extraction/precipitation procedure was repeated one more time before the RNA was used for cDNA synthesis.

cDNA Synthesis & Polymerase Chain Reaction

Conditions for cDNA and PCR were essentially as described by Erlich (18). First strand cDNA synthesis was carried out using 1-2µg total cellular RNA, 100 pmoles random hexamers (primer), 0.25 mM dNTP's, 0.1 volume 10X PCR buffer (KCl; 0.5M, Tris-8.3; 0.2M, MgCl; 25mM, BSA; 1mg/ml) in a 20 µl volume. The RNA solution was

incubated at 95°C for 1 minute then quick cooled on ice before 200 units of MuLV Reverse transcriptase (Gibco BRL) was added. The reaction was incubated at room temperature for 10 minutes followed by 37°C for an additional 60 minutes. The entire RTase reaction was used in the subsequent PCR step. Additional 10X PCR buffer and dNTP's (1 mM) were added to the RTase reaction as well as 0.25μM of each of the two primers (Vβ6 and Cβ -sequences shown in Figure 4.1). The final reaction volume was 100 μl to which 4 units of Taq polymerase (Promega) was added. The reaction was overlayed with parafin oil to prevent evaporation. Temperature cycling steps included a denaturation step (92°C; 2 min.), an annealing step (55°C; 1 min.) and and extention step (72°C; 1 min.). This cycle was repeated 35 times. A portion of the PCR reaction was run on a 2% agarose gel and the DNA band was purified.

Cloning of PCR Products

The purified PCR product was phosphorylated with polynucleotide kinase (PNK) and blunt-ended with T4 DNA polymerase before being ligated into the SmaI site of pUC 18. Reaction conditions were essentially as described in Maniatis et al. (17). Ligation reactions were transformed into competent TB-1 *E. coli* and plated onto LB agar plates containing ampicillin and X-gal. The bacteria was incubated at 37°C overnight. Recombinant (white) colonies were picked for sequencing.

Double stranded Sequencing

Plasmid mini-preps were done on recombinant colonies. Overnight cultures were grown in 10 ml of TB,"Terrific Broth" (for 1 litre- KH_2PO_4:2.31g, K_2HPO_4:12.5g, Tryptone:12g, Yeast Extract:20g, glycerol:4ml). Bacteria were pelleted by centrifugation at 3000 rpm for 10 min. The pellet was resuspended in 250 µl glucose solution (glucose:250mM, Tris pH8:25mM, EDTA:10mM) and transfered to an Eppindorf tube. Following the addition of 500 µl NaOH/SDS solution (NaOH:0.2M, SDS:1%) the contents of the tube were mixed by inversion for 3 min. Next, 250 µl of 5M potassium acetate was added and the tube was placed on ice for 3-5 min. The sample was centrifuged at 12,000 rpm for 3 min to pellet the debris. The supernatant was transfered to another Eppindorf tube containing 500 µl isopropanol and centrifuged for 5 minutes to precipitate the plasmid DNA. The DNA pellet was resuspended in 20 ml TE (Tris pH8:10mM, EDTA:1mM). Double stranded DNA sequencing was done according to the guidelines given in the Sequenase DNA sequencing kit (United States Biochemicals). A nested Cβ primer and/or the Vβ6 oligonucleotide were used in place of the universal primer in the sequencing reactions (shown in Figure 4.1).

Results

The BALB/c derived poly-18 specific T cell hybridomas expressing Vβ6 were assessed for their ability to respond to Mls-1^a on CBA/J splenocytes. Summary table 4.1 shows the results from two such experiments. Hybridomas B2, B4, B5, B11 and B17 all respond to the CBA/J splenocytes. No response was seen in hybridomas B3, B8, and B14, despite expression of Vβ6. The TCRα chain or alternatively the β chain junctional diversity could be responsible for the inability of these hybridomas to recognize Mls-1^a. The Vα gene usage in these hybridomas was determined using V gene family specific probes in a Northern analysis (taken from Chapter 2) The results are also listed in Table 4.1. Vα8, Vα1 and an unknown Vα are being used by the Mls-1^a nonreactive hybridomas. These Vα genes are not expressed in the Mls-1^a reactive hybridomas. The junctional diversity and Jβ usage associated with Vβ6 in these hybridomas are shown in Table 4.1 (taken from Chapter 2). T cell hybridomas B2, B4 and B5 all use Jβ2.4 with the same junction sequences. The remaining Mls-1^a reactive hybridomas, B11 and B17, use Jβ1.2 and 1.3 respectively. The 3 Mls-1^a nonreactive hybridomas, B3, B8 and B14 use Jβ1.3, 2.3 and 2.7 respectively. The junctional diversity associated with Vβ6 and Jβ1.3 in B3 and B17 differs.

Two T cell lines specific for poly-18 were generated from separate pools of immunized DBA/2 mice. Each line was carried on poly-18 for 4-6 weeks. Proliferation assays demonstrated reactivity to poly-18 while DBA/2 spleen cells alone failed to elicit a response

(Table 4.2). These lines did not show any reactivity to PPD when tested (data not shown). Thus the lines were poly-18 specific without a detectable Mls-1^a autoreactive component.

Total RNA prepared from these two lines was used to generate first strand cDNA. Two separate reverse transcriptase (RTase) reactions from each line were done and the subsequent sequence data pooled for each line so that any artificial skewing by this enzymatic step could be overcome. The products of the RTase reactions were amplified using TCR Vβ6 and Cβ specific oligonucleotides (Fig 4.1) in a polymerase chain reaction. The DNA products were approximately 190 bp in length as predicted from the published sequences of Vβ6, D,J and Cβ (19-22). After blunt-end cloning of the Vβ6 amplified DNA into the Sma I site of pUC18, colonies were picked at random for double stranded sequencing. A nested Cβ oligonucleotide (Fig 4.1) was used as primer for the sequencing. Sequence data from each cell line is shown in Figure 4.2. Only the 3' portion of the Vβ6 sequence and the 5' portion of the Jβ's are shown. Amino acid sequences of the junction region are shown above the DNA sequence. The number of times that each clone was observed is shown in the columns under each cell line. From the 2 DBA/2 anti-poly-18 T cell lines, a total of 57 sequences are shown, 35 from #1 and 22 from #2. Taken together, 19 different rearrangements have been identified, utilizing 8 of the 12 Jβ's. While 21 different rearrangements are shown in the figure, 2 of these clones may be derived from polymerase errors as they differ from two other sequences by only one base (single base differences underlined in figure 4.2).

Discussion

The observation that 3 of the 8 BALB/c Vβ6+ poly-18 specific T cell hybridomas fail to respond to Mls-1a suggests two things. Firstly, not all Vβ6 T cells can recognize Mls-1^a. This finding is consistent with earlier studies (4). Thus while the correlation between Vβ6 expression and Mls-1^a reactivity is clear, there appears to be a fraction of Vβ6 bearing T cells that do not respond to Mls-1^a. Secondly, the Vβ6 repertoire used in the poly-18 response overlaps with the Mls-1^a reactive repertoire rather than being a subset of it. Therefore, immunization with poly-18 should allow clonal expansion of some Vβ6 T cells that escape deletion in a Mls-1^a strain of mouse.

After successfully generating poly-18 specific T cell lines from DBA/2 mice (Mls-1a), we analysed the junctional diversity and J region usage associated with Vβ6 in these T cells. Three aspects of these results are noteworthy. First, the very existence of $V\beta6^+$ T cells in the periphery of DBA/2 mice. Analysis with Vβ6 specific monoclonal antibodies couldn't address whether the thymic deletion of these cells in Mls-1^a strains was complete (4). Something about the Vβ6 T cell receptors on the cells we isolated from DBA/2 mice reduces the inherent Mls-1^a reactivity. Secondly, these $V\beta6^+$ T cells which are not deleted in the DBA/2 thymus can participate in an antigen specific response. Studies using TCR Vβ8.1 transgenic mice have demonstrated that clones that escape deletion in the thymus can become anergic, probably due to peripheral tolerization (14). Our own data suggests that some Vβ6 expressing T cells escape both central deletion and peripheral tolerization because they do not

recognize Mls-1^a. Finally, the extent of the diversity seen with respect to junction sequences and J region usage in these Vβ6$^+$ T cells is striking. The fact that 17 of the 19 DBA/2 Vβ6 sequences have been identified in our analysis of the BALB/c anti-poly-18 T cell repertoire supports the antigen specificity of these T cell receptors. Since the sequences were derived from bulk T cell lines, it is not possible to say definitively that the TCR's cloned by PCR were specific for poly-18. It should be noted that the DBA/2 anti-poly18 Vβ6 repertoire represents only a fraction of the estimated Vβ6 repertoire in the BALB/c anti-poly-18 T cell response. This relationship is expected given the documented deletion of Vβ6$^+$ thymocytes in Mls-1^a mice(4). An accurate estimation of the proportion of Vβ6 T cells deleted in the DBA/2 mouse is not possible using the results here.

Junctional sequences from 2 of the 3 Mls-1^a nonreactive BALB/c T cell hybridomas (B8 and B14) are also present in the DBA/2 T cell lines. The selection of these junction regions in the DBA/2 suggests a possible role in conferring nonreactivity to Mls-1^a. However, one example of a junction region that is associated with Mls-1^a reactivity in the hybridomas (B11) is seen in the DBA/2 T cell line analysis. This argues for a potential role for the α chain in conferring nonreactivity to Mls-1^a in some TCR's.

There doesn't appear to be any obvious skewing toward or away from any particular Jβ in the DBA/2 Vβ6 repertoire. For example, Jβ2.3, 2.5 and 2.7 are all over-represented in terms of frequency and number of junction sequences in both the BALB/c and DBA/2 Vβ6 repertoires. Three Jβ's used infrequently in the BALB/c repertoire (Jβ1.1, 1.6 and 2.2) are not seen in these DBA/2 lines.

Their absence may have more to do with their low frequency in the poly-18 response than their role in Mls-1^a reactivity. The effects of thymic deletion appears to reduce the number of permissible Vβ6 junction sequences for most of the J regions. The junction regions are potentially involved in allowing threshold affinities between the $V\beta6^+$ receptor and the Mls-1^a antigen. The role of individual J regions may depend on the junctional diversity associated with them. Certain rare junction/J region combinations could act to disrupt contact leading to an escape from deletion. Most combinations presumably either increase the association or have a net neutral effect and are subsequently deleted in these mice.

The possibility remains that particular TCRα chain(s) may interfere with Mls-1^a reactivity in some situations. We identified three Vβ6+ T cell hybridomas in the BALB/c anti-poly-18 repertoire analysis that were not Mls-1^a reactive (Table 4.1). Northern analysis showed that 3 different Vα genes were being used; Vα1 and Vα8 and an unknown Vα. This suggests that a single α chain is unlikely to be the determining factor in disrupting Mls-1^a reactivity. TCRα chains of more Vβ6+ Mls-1^a non-reactive T cells need to be analysed to critically assess the role of the α chain in Mls-1^a recognition.

From this data it is clear that deletion of Vβ6 thymocytes in Mls-1^a strains is an incomplete process. This failure to be deleted does not appear to be associated with a particular Jβ region. Neither does there appear to be a single α chain that is associated with Mls-1^a nonreactivity in Vβ6 T cells. The apparent complexity of the Vβ6 TCR's that do not recognize Mls-1^a may indicate that both junctional diversity in the β chain and/or the α chain can disrupt the

interaction. Some of the Vβ6 cells that emerge from the DBA/2 (Mls-1^a) thymus are clearly able to participate in antigen specific T cell responses. The fact that these cells have not been deleted and are not anergic implies that they have not interacted with the Mls-1^a antigen in the thymus. Part of the difficulty is the fact that the antigenic entity of Mls-1^a is not described. Whether Mls-1^a binds the antigenic cleft of the TCR or interacts directly with the Vβ region is not known (23). There is mounting evidence for the primary role of the Vβ region in these responses. Pullen et al. (24) have identified amino acids in the Vβ8.1 gene that are critical for Mls-1^a reactivity. These amino acids are thought to reside well away from the TCR's antigen binding cleft. The data discussed in this communication does not speak directly to this issue but does argue for the involvement of other components of the TCR in some aspects of Mls-1^a recognition. This may take the form of either altering the conformation of the TCR such that the relevant sites on the Vβ are not accessible to Mls-1^a, or be due to a direct interaction of Mls-1^a with regions of the antigen binding cleft of the TCR.

If the TCR recognition of superantigens is discussed in the context of other antigen systems, a certain dichotomy becomes apparent. Superantigens by definition, are recognized primarily by elements in the Vβ region of the TCR. Largely by inference, the junctional diversity, Jβ usage and the TCR α chain play a secondary role. We have shown that recognition of Mls-1^a by Vβ6 T cells is not solely dependent on the Vβ. Therefore the role of individual components of the TCR in Mls-1^a recognition must be viewed in a relative context. The antigen systems that have been used to

characterize the TCR interactions with antigen/MHC have given a variety of reactivity patterns. The well characterized systems of cytochrome c (25-29), myelin basic protein (30-32), and the hapten TNP on H-2K^b (33,34) all show evidence for the critical involvement of elements in both the α and β chains of the TCR. The response to pABA (35), myoglobin (36,37) and poly-18 (Chapters 2 and 3) represent intermediates between the response patterns for other antigens and the superantigens. The myoglobin response is dominated by Vβ8.2 with limited junctional diversity and Jβ region usage. The α chains that can pair with the dominant Vβ gene are quite diverse. The T cell response to pABA is dominated by the use of Vα3. A number of lines of evidence suggest that the Vα3 segment is responsible for pABA reactivity on at least 3 MHC molecules. Poly-18 specific T cells that use Vβ6 or Vα11 may rely heavily on the dominant V genes alone for reactivity. The junctional diversity and Jβ region usage as well as the TCR α chain are all very diverse. This reactivity pattern parallels some features of the T cell response to superantigens. This suggests that the Vα11 and Vβ6 may be playing a primary role in reactivity to poly-18 while the other components of the TCR are secondary, but still involved in determining antigen fine specificity. Thus their are a number of ways that the TCR can interact with antigens depending on the relative involvement of different regions of the TCR with the antigen/MHC complex.

Table 4.1. BALB/c poly-18 specific Vβ6+ T cell hybridomas summary table: V gene usage, Vβ6 junctional diverstiy and Mls-1^a reactivity.

T cell Hybrid	V gene usage[1] Vα	Vβ	Vβ6 Junctional Diversity and J region Usage[2] Vβ6	Dβ	Jβ		Reactivity with CBA/J Splenocytes (x 10^3cpm)[3] Exp#1 -	Exp#1 +	Exp#2 -	Exp#2 +	Mls-1a Reactive
B2	5	6	C A S S TGT GCC AGC AG	T	M G T R ATG GGG ACA AGA	N T L Y AAC ACC TTG TAC- 2.4	3	39	1	19	+
B4	-	6	C A S S TGT GCC AGC AG	T	M G T R ATG GGG ACA AGA	N T L Y AAC ACC TTG TAC- 2.4	1	45	1	40	+
B5	5	6	C A S S TGT GCC AGC AG	T	M G T R ATG GGG ACA AGA	N T L Y AAC ACC TTG TAC- 2.4	1	12	1	9	+
B11	7	6	C A S S TGT GCC AGC AG	T	I G Q ATA GGA CAA	N S D Y T AAC TCC GAC TAC ACC- 1.2	1	12	1	38	+
B17	ND	6	C A S S TGT GCC AGC AG	T	R D G H S CGG GAC GGA CAT TCT	G N T L Y GGA AAT ACG CTC TAT- 1.3	1	61	1	19	+
B3	8	6	C A S S TGT GCC AGC AG	C	Q G Q L CAG GGA CAG CTT	S G N T L Y AGT GGA AAT ACG CTC TAT- 1.3	1	1	1	1	-
B8	-	6	C A S R TGT GCC AGC AG	G	G L G P GGA CTG GGG CCT	S A E T L Y AGT GCA GAA ACG CTG TAT- 2.3	1	1	1	1	-
B14	1	6	C A S S TGT GCC AGC AG	T	I S F ATA TCC TTT	Y E Q Y TAT GAA CAG TAC 2.7	1	1	1	1	-

1 V gene usage for each T cell hybridoma was taken from table 2.2

2 Junctional diversity shows the 3' end of Vβ6 and 5' ends of the Jβ's. The Jβ used by each hybridoma is given. Single letter amino acid code is given above each codon.

3 Reactivity of the hybridomas with (+) and without (-) CBA/J (Mls-1^a) splenocytes was determined in a ^{3}H-thymidine uptake assay. Results are rounded up to the nearest 10^3.

Table 4.2. DBA/2 poly-18 specific T cell lines proliferation assay[1]

T CELL LINE	APC*	Poly-18	cpm	(+/-SD)
DBA/2-P18#1	+	-	6093	(752)
	+	+	115490	(23977)
DBA/2-P18#2	+	-	3098	(-)
	+	+	79239	(16917)

* Irradiated spleen cells (3000 rad) from DBA/2

[1] Reactivity of T cells from each DBA/2 line in the presence (+) or absence (-) of poly-18 is shown. All assays were done in duplicate except for P18#2 without poly-18.

Cβ(5')	antisense	5'CAAGG	AGACC	TTGGG	TGGAG	TCAC3'
Cβ(3')	antisense	5'TCTCT	GCTTT	TGATG	GCTCA	AACA3'
Vβ6	sense	5'GGCGA	TCTAT	CTGAA	GGCTA	TGAT3'

Figure 4.1. Oligonucleotides used for PCR amplification and sequencing of Vβ6 junction regions.

Vβ6	Dβ	Jβ	DBA/2 Cell Line #1	#2	
C A S R	T G S	N S D Y T			
TGT GCC AGC AG	A ACA GGA TCA	AAC TCC GAC TAC ACC-1.2	1	-	*
C A S S	I G Q	N S D Y T			
TGT GCC AGC AG	T ATA GGA CAA	AAC TCC GAC TAC ACC-1.2	-	3	*
C A S S	T G T G T	G N T L Y			
TGT GCC AGC AG	C ACG GGG ACA GGG ACT	GGA AAT ACG CTC TAT-1.3	1	-	
C A S S	R T L	S N E R L F			
TGT GCC AGC AG	T CGG ACA CTT	TCC AAC GAA AGA TTA TTT-1.4	1	3	*
C A S S	L R G L	S N E R L F			
TGT GCC AGC AG	T CTC AGG GGG CTT	TCC AAC GAA AGA TTA TTT-1.4	1	-	*
C A S S	M G V	Y A E Q F			
TGT GCC AGC AG	T ATG GGG GTC	TAT GCT GAG CAG TTC-2.1	1	-	
C A S S	R G L R	A E T L Y			
TGT GCC AGC AG	T AGG GGA CTG AGG	GCA GAA ACG CTG TAT-2.3	1	2	*
C A S R	G L G P	S A E T L Y			
TGT GCC AGC AG	G GGA CTG GGG CCT	AGT GCA GAA ACG CTG TAT-2.3	1	-	*
C A S	M G L G P	S A E T L Y			
TGT GCC AGC A	IG GGA CTG GGG CCT	AGT GCA GAA ACG CTG TAT-2.3	1	-	
C A S S	I A H R V	A E T L Y			
TGT GCC AGC AG	T ATA GCC CAC AGG GTC	GCA GAA AGC CTG TAT-2.3	4	-	*
C A S S	L R D R R	A E T L Y			
TGT GCC AGC AG	T CTC CGG GAC AGG CGT	GCA GAA ACG CTG TAT-2.3	-	1	*
C A S S	R G G P	S Q N T L Y			
TGT GCC AGC AG	T CGG GGG GGC CCT	AGT CAA AAC ACC TTG TAC-2.4	3	-	*
C A S R	D R	D T Q Y			
TGT GCC AGC AG	G GAC AGG	GAC ACC CAG TAC-2.5	5	3	*
C A S	K D R	D T Q Y			
TGT GCC AGC A	AG GAC AGA	GAC ACC CAG TAC-2.5	-	2	*
C A S S	I R	D T Q Y			
TGT GCC AGC AG	T ATA CGG	GAC ACC CAG TAC-2.5	3	-	*
C A S S	P L G G R	D T Q Y			
TGT GCC AGC AG	T CCA CTG GGG GGC AGC	GAC ACC CAG TAC-2.5	1	-	*
C A S S	I A V M	Y E Q Y			
TGT GCC AGC AG	T ATA GCT GTC ATG	TAT GAA CAG TAC-2.7	6	-	*
C A S S	I A V I	Y E Q Y			
TGT GCC AGC AG	T ATA GCT GTC ATA	TAT GAA CAG TAC-2.7	1	-	
C A S S	I S F	Y E Q Y			
TGT GCC AGC AG	T ATA TCC TTT	TAT GAA CAG TAC-2.7	-	2	*
C A S	K Q G P	S Y E Q Y			
TGT GCC AGC A	AA CAG GGT CCC	TCC TAT GAA CAG TAC-2.7	3	1	*
C A S S	R L G A I	Y E Q Y			
TGT GCC AGC AG	T CGA CTG GGG GCC ATC	TAT GAA CAG TAC-2.7	1	5	*
			35	22	

* Clones seen before in the BALB/c anti-Poly-18 T cell repertoire

Figure 4.2 Junctional diversity and J region usage in Vβ6 sequences from DBA/2 poly-18 specific T cell lines, #1 and 2.

Sequences are organized according to Jβ usage. The 3' end of Vβ6 and 5' ends of the Jβ's are shown. The Jβ usage is given for each clone. Single letter amino acid code is given above each codon. The number of times that each clone was identified is shown in the columns uner each cell line. Single base differences between pairs of clones are underlined in the figure. These may represent artifacts of amplification or sequencing.

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Chapter 5

General Discussion and Conclusions

The work discussed in this thesis has focused on the T cell response to poly-18 in BALB/c and DBA/2 mice. The objectives of this study were to examine how the T cell receptor interacts with the antigen-MHC complex. The approach involved a characterization of the T cell receptors used in the BALB/c poly-18/I-A^d response. This analysis included a series of steps, each intended to increase the size and scope of the study. The results of the analysis of the BALB/c T cell repertoire to poly-18 prompted the decision to characterize part of the DBA/2 T cell response to poly-18.

BALB/c T cell repertoire to poly-18

Initially, cDNA cloning and sequencing was done on individual T cell hybridomas from BALB/c whose antigen fine specificity had been characterized. Three TCRα and β chains were involved in the response were identified this way. Unfortunately, no common TCR gene segments in either chain were being used by these three hybridomas. Neither were there any apparent conserved amino acids that could be implicated in conferring poly-18 reactivity.

A panel of TCR V gene DNA probes either generated in our cDNA analysis or kindly made available to us by the laboratory of Dr. L. Hood (Caltech, Pasedena, Ca), allowed us to broaden the scope of

the study. Northern analysis using the V region probes on an additional 13 poly-18 specific T cell hybridomas from BALB/c was done. Southern analysis of TCR rearrangement patterns for Vα1 and Vβ1 was necessary because the fusion partner, BW5147, expresses both of these V regions. Two V region specific monoclonal antibodies were available for use in a FACS analysis to confirm results of the northern analysis. These were 44-22-1 and F23.1, specific for Vβ6 and the Vβ8 family respectively. TCR V gene usage in the 16 T cell hybridomas revealed 2 important features of the T cell response to poly-18 (Chapter 2). Firstly, either Vβ6 or Vα11 were being expressed in 12 out of 16 (75%) hybridomas tested. Secondly, a large number of TCRα chains could associate with Vβ6, and similarly, a large number of β chains could associate with Vα11. These two observations taken together, suggested to us that the dominant V gene usage of Vα11 and Vβ6 could be due to those V genes playing a primary role in conferring reactivity to poly-18 as presented by I-A^d. If this was true, what was the role of the junctional diversity and J regions associated with the dominant V genes? Were just the dominant V genes being conserved in these TCR's or were a limited number of junctions sequences also important in conferring poly-18 reactivity. Using the polymerase chain reaction (PCR), we were able to specifically amplify and clone Vα11 and Vβ6 junction sequences from the appropriate hybridomas. The results of this analysis indicated that J regions and junction sequences associated with both Vα11 and Vβ6 were not being conserved.

To confirm the trend seen in the analysis of Vα11 and Vβ6 junctional diversity from the hybridomas, we needed to increase our

sample size. It was apparent that the junctional diversity associated with each of the dominant V genes could be quite large. To accomplish this, we switched to analysing poly-18 specific bulk T cell lines from BALB/c that had been carried in culture for 4-6 weeks. Using PCR technology for the analysis of Vα11 and Vβ6 junction regions found in mixed populations of T cells allowed us to identify many junction sequences potentially participating in the poly-18 response (Chapter 3). We assumed that most of the sequences isolated from the poly-18 specific T cell lines were for TCR's involved in the response. The trend seen in both Vα11 and Vβ6 chains was the same. The Vα11 gene was found rearranged to at least 16 Jα regions in the two BALB/c poly-18 specific T cell lines assessed. In the Vβ6 analysis of 6 separate T cell lines, 11 of the 12 possible Jβ regions were detected. The junctional diversity flanking the Dβ region in these TCR's was extremely diverse. As many as 11 different junction sequences were seen with individual Jβ regions. Our notion that reactivity to poly-18 might be conferred largely by the dominant V genes was supported by the junction analysis. Rather that a whole chain of the TCR being associated with antigen reactivity, it was conceivable that Vα11 and Vβ6 were each capable of conferring poly-18 reactivity.

The interpretation of our data stems from the view that immune responses are shaped by strong selective pressures at many levels. In much the same way that evolution affects whole organisms, the selective pressures in the immune system affect whether T cells will live or die. Most of the selection is mediated through the TCR. The size and complexity of the peripheral T cell

repertoire is determined by selection events in the thymus (see Chapter 1). Only those thymocytes that are not negatively selected, yet undergo positive selection, will become part of the peripheral T cell repertoire. These selection events are clearly dependent on TCR specificity. Mature T cells are selected for clonal expansion by their ability to respond to antigen/MHC complexes. The components of the TCR that are important in conferring reactivity toward a given antigen/MHC complex will be selected too. As discussed earlier, many of the well characterized antigen systems show evidence of this selective pressure through the limited number of TCR elements used. This is true for T cell responses to cytochrome c (1-6), myelin basic protein (7-9), and TNP/H-2K^b (10,11). In these systems, strong selective pressure to use a limited number of TCR elements in the antigen specific response reflects the importance of those elements in conferring reactivity to the antigen. A very different V gene usage pattern has emerged from the analysis of a class of antigens, now referred to as superantigens. These include an unknown self antigen plus I-E (12,13), Mls-1^a (14-16), Mls-2^a (17,18) and a number of Staphylococcal enterotoxins (19,20). The T cell response to these antigens is dictated almost exclusively by different Vβ regions. Consequently, there is little selective pressure to limit the use of any of the other components of the TCR. Thus two very different V gene usage patterns are apparent. One that uses a limited number of TCR gene segments and junctional diversity. The other that uses one or a few V regions with almost any combination of other TCR elements. These V gene usage patterns reflect the functional significance of the different elements of the TCR in the different antigen systems.

In the poly-18 system, we suggest that the lack of selective pressure to use a limited number of elements of the TCR with either Vα11 or Vβ6 reflects the secondary role of those other elements in conferring reactivity to poly-18. A few other nominal antigen systems are similar to poly-18 in their pattern of V gene usage. The T cell response to the hapten pABA is dominated by the use of Vα3 (21). At least 2 Jα regions and 3 Vβ regions were associated with Vα3 in 4 pABA specific T cell clones. The authors provide functional evidence that Vα3 is largely responsible for conferring pABA reactivity on as many as 3 different MHC molecules. The myoglobin system may represent an intermediate between poly-18 and pABA responses and those responses that show strong selection for all elements in the TCR. In six myoglobin specific T cell clones expressing Vβ8.2, 2 Jβ regions, 3 Vα regions and 4 Jα regions were detected (22,23). When all of the antigen systems are categorized according to their TCR gene usage pattern, a continuum of response patterns are seen. This continuum ranges from near complete dependence on single V regions (superantigens), to strong selection for all elements of the TCR (cytochrome c, myelin basic protein, TNP/H-2K^b), with a number of potential intermediates that show dominant use of certain V regions with relatively less selection for other elements (poly-18, pABA, myoglobin). At present no superantigen has been associated with a Vα region. The predominance of Vα11 in part of the poly-18 response, and Vα3 in the pABA response suggests that either Vα or Vβ regions can play a primary role in conferring reactivity toward an antigen.

A number of factors might account for this range of reactivity patterns. One possibility is that the TCR can interact with antigen-MHC complexes in a variety of ways. TCR modelling studies suggest that the first and second hypervariable regions on each chain (which are encoded by the Vα and Vβ regions) contact amino acid residues along the 2 α-helices of the MHC molecule (24,25). Whether the TCR Vα and Vβ regions always contact the same α-helix is not known. There is potential to interact with different regions of the MHC along the length of the groove or rotate in space to make contact with different elements of the TCR. The other scenario that could account for the range of reactivity patterns involves the nature of the interactions between antigen and MHC. Within the MHC groove, antigen could bind in a number of ways. This might account for the complexity of some antigen specific T cell repertoires. Flexibility in how peptides of poly-18 are bound by MHC has been reported (26). Alternatively, some antigens might bind MHC outside the groove. The strong association between superantigens and certain Vβ regions may reflect this type of interaction. Dellabona et al. have used single amino acid substitutions at 30 positions along I-A_α^k to demonstrate the differential presentation of peptides and superantigens (27). Their work does support the premise that superantigens do bind Ia outside the groove. Whether other antigen types can also bind outside the groove is not known.

DBA/2 Vβ6+ T cell receptor repertoire to poly-18 (and Mls-1^a)

The observation that Vβ6 was used extensively in the T cell response to poly-18 in BALB/c (Mls-1^b) coincided with the characterization of the T cell response to Mls-1^a (15). Part of the T cell response to this superantigen was dictated by Vβ6. Near complete deletion of Vβ6 thymocytes in Mls-1^a strains was noted and most randomly selected Vβ6 T cells (11/14) reacted specifically with Mls-1^a. This implied that other elements of the TCR were playing a greatly diminished role in this response. Whether the deletion process was complete in these mice was difficult to ascertain using monoclonal antibodies. Also, the possibility existed that if some Vβ6 T cells did escape deletion, they may be rendered anergic by peripheral tolerization mechanisms. Characterization of the Mls-1^a reactivity of the Vβ6 T cell hybridomas from BALB/c revealed 3 nonreactive hybridomas. Three different Vα genes were being used by the 3 Mls-1^a nonreactive Vβ6 hybridomas, suggesting that a few Vα genes were not responsible for disrupting Mls-1^a reactivity. Also, the Vβ6 repertoire to poly-18 evidently overlapped with the Vβ6 repertoire to Mls-1^a, rather that being a subset of it. This finding and the large number of TCRα chains, Jβ's and junctional diversity associated with Vβ6 in the poly-18 response led us to speculate that this might be an appropriate antigen to amplify rare Vβ6 T cells in strains of mice that were thought to delete their entire Vβ6 compartment. Working with antigen specific T cell lines

removed the chance of isolating anergic clones like those found in Vβ8.1 transgenic mice (28).

Two T cell lines specific for poly-18 were generated from DBA/2 mice. As these mice express Mls-1^a, the vast majority of their Vβ6 T cells are deleted during thymic maturation (15). FACS analysis with a Vβ6 specific monoclonal antibody (44-22-1) revealed the presence of Vβ6 T cells in the lines (data not shown). Yet these lines did not respond to Mls-1^a on DBA/2 spleen cells. The response of the Vβ6 fraction of the lines could not be assessed separately.

To assess the Vβ6-Jβ region usage and junctional diversity associated with these Mls-1^a nonreactive T cell lines, we amplified and cloned junction sequences using PCR. A total of 57 Vβ6 junction sequences were generated from the two poly-18 specific cell lines from DBA/2. Nineteen different clones were found in the analysis using 8 of the 12 possible Jβ regions. Almost all of the clones (17/19) were sequences that had been isolated in the BALB/c cell line analysis. This supported the assumption that these sequences were from TCR's participating in the poly-18 response. Also, it supported the pattern of Vβ6 junctional diversity seen in the poly-18 T cell response. Since it is known that this strain of mouse deletes a significant portion of its Vβ6 repertoire, it only follows that the poly-18 specific Vβ6 repertoire in DBA/2 would be narrower than the BALB/c Vβ6 repertoire. A similar use of Jβ regions was noted in the DBA/2 and BALB/c Vβ6 repertoires. Those Jβ's found in a number of rearrangement patterns in the BALB/c response (Jβ2.3, 2.5 and 2.7) were likewise the most numerous in the DBA/2 response. Those Jβ regions absent or found in only 1 or 2 rearrangement patterns in the

BALB/c response (Jβ1.1, 1.5, 1.6 and 2.2) were absent from the DBA/2 response.

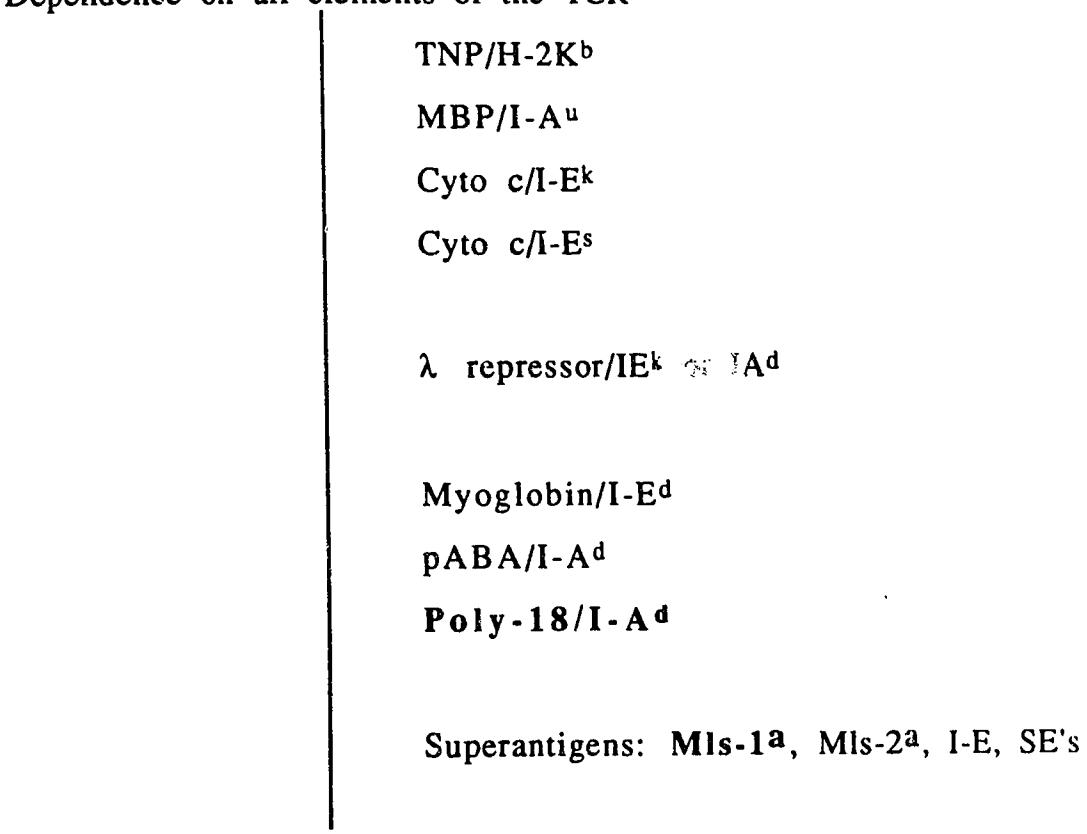
The results of the DBA/2 analysis indicate a number of things about Vβ6 recognition of Mls-1^a and negative selection. The very existence of these Vβ6 sequences from a DBA/2 cell line indicates that clonal deletion of Vβ6 thymocytes is incomplete. Some Vβ6 T cells are allowed to exit the thymus and form part of the peripheral T cell repertoire. The ability of these cells to participate in an antigen specific response means that they are not anergic. What component of the $V\beta6^+$ TCR is responsible for disrupting Mls-1^a reactivity? Clonal deletion of Vβ6 thymocytes results in an apparent overall reduction in the number of different junction sequences associated with each Jβ in the DBA/2. No skewing toward or away from any particular Jβ can be noted, implying that the Jβ's themselves are not responsible for disrupting Mls-1^a reactivity. The fact that 3 different Vα genes are associated with Mls-1^a nonreactivity in the BALB/c Vβ6 hybridomas suggests that particular Vα genes do not always disrupt Mls-1^a reactivity. The Vβ6 junction sequences associated with 2 of the 3 Mls-1^a nonreactive hybridomas are seen in the DBA/2 lines. The selection of these junction sequences may indicate their involvement in disrupting Mls-1^a reactivity. The possibility exists that rare Vβ6 junction sequences do interfere with the TCR's ability to bind Mls-1^a. Amino acids in Vβ8.1 critical for Mls-1^a reactivity have been identified (29). These amino acids are thought to reside well away from the conventional antigen binding cleft of the TCR. This supports the notion that superantigen recognition mediated by Vβ regions of the TCR is different from

recognition of other antigen types. Our work characterizing the exceptions to the Vβ6/Mls-1^a association suggests a role for the junction sequences in some interactions. This may come in the form of altering conformation slightly, thereby changing the ability of Vβ6 to interact with Mls-1a. Alternatively, the antigen binding cleft of the TCR may be involved in Mls-1^a recognition. The intricacies of this debate remains to be resolved.

T cell recognition

The work described in this thesis has approached the topic of antigen recognition from two perspectives. One involving the T cell response to a peptide antigen (poly-18) and the other the recognition of a superantigen (Mls-1a). The characterization of the T cell response to poly-18 revealed a TCR gene usage pattern similar in some ways to one expected for a superantigen. On the other hand, the characterization of the exceptions to the Vβ6/Mls-1^a correlation suggested that β chain junctional diversity can influence superantigen recognition. Taken together, these two observations reduce the apparent difference between nominal antigen and superantigen recognition. The image of a continuum of response patterns as reflected by TCR gene usage patterns emerges. The TCR gene usage patterns of a variety of antigen responses are shown in figure 5.1. The antigen systems are placed along a continuum, ranging from an apparent dependence on all elements of the TCR, to a dependence on individual V regions for responsiveness.

Figure 5.1 Continuum of TCR gene usage patterns.



Unfortunately, our ability to predict what type of response pattern an antigen will generate is still very poor. Eventually this may be overcome as we come to understand the range of interaction that can take place between the TCR and the antigen-MHC complex.

Future directions

This study has generated as many questions as it has attempted to answer. There are a number of features of the T cell response to poly-18 that remain to be addressed. The extent of the

correlation between poly-18 reactivity and Vβ6 or Vα11 expression should be further characterized. A number of approaches can be used to assess this. Randomly chosen T cells that express these V genes but are specific for other antigens can be tested for their ability to respond to poly-18. This would provide an estimate of the proportion of T cells expressing these V genes that would crossreact on poly-18/I-A^d. If this analysis confirms the correlation between poly-18 reactivity and expression of these V genes, more direct experimental approaches would be warranted. Transfection experiments could potentially show transfer of specificity through single chain transfers.

We are suggesting that the T cell response to poly-18 differs from many other peptide antigens. The possibility that poly-18 binds the MHC outside the antigen binding groove needs to be explored. Since poly-18 peptides do not require processing (30), competition experiments could be designed to test where it binds. Peptides of poly-18 that bind outside the groove should not be able to compete for sites with other peptides that are thought to bind inside the groove. Mutagenesis of I-A^d at various positions could be used to show differential binding of poly-18 and other peptides. This approach has been used successfully to support the notion that superantigens bind outside the groove (27). Alternatively, mutagenesis of the TCR Vβ6 or Vα11 at positions shown to be important in superantigen recognition might extend the parallels drawn between the two systems by this study (29).

The involvement of junction sequences in the recognition of Mls-1^a by Vβ6 T cells has been suggested by this study. Direct

demonstration of the influence of junction sequences might be accomplished now that a number of sequences associated with nonreactivity have been identified. Transfection of Vβ6 β chain genes that differ only in the junction sequences could accomplish this goal. Analysis of the α chains of more Mls-1^a nonreactive Vβ6$^+$ T cells might indicate whether certain segments of the α chain are more likely to disrupt Mls-1^a recognition.

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